NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF INTRAMURAL RESEARCH

SECOND ANNUAL
FELLOWS RETREAT

JUNE 24-25, 2004
Airlie Conference Center

Organizing Committee

Dr. Fraser Brown,Laboratory of Cell Biology
Dr. Martin Crook, Cardiovascular Branch
Dr. Sandeep Dave, NHLBI FELCOM Representative
Dr. Luca Di Noto, Laboratory of Biochemistry
Dr. Cheryl Hawkins, Laboratory of Biophysical Chemistry
Dr. Heather Jones, Pulmonary-Critical Care Medicine Branch
Dr. Mihaly Kovacs, Laboratory of Molecular Cardiology
Dr. Jinhee Lee, Laboratory of Cell Signaling
Dr. Jun Lu, Hematology Branch
Dr. Ryan Morris, Laboratory of Kidney & Electrolyte Metabolism
Dr. Paola Pozzi, Laboratory of Biochemical Genetics
Dr. Ivan Rosas, Pulmonary-Critical Care Medicine Branch
Dr. Emily Rothstein, Laboratory of Cardiac Energetics
Dr. Phillip Scheinberg, Hematology Branch
Dr. Hymavathi Tirupati, NHLBI FELCOM Representative
Dr. Diana Walker, Laboratory of Developmental Biology
Dr. Kevin Welch, Laboratory of Molecular Immunology,
Dr. Andrew Wragg, Cardiovascular Branch

With Special Help from
NHLBI Office of Science and Technology
DIR Office of Education

June 24

9:00 - 10:00 **Continental Breakfast - Federal Room**

10:00 - 10:15 **Welcoming Remarks - Federal Room**
Dr. Martin Crook, Retreat Organizing Committee
Dr. Barbara Alving, Acting Director, NHLBI
Dr. Robert Balaban, Scientific Director, LRP, DIR, NHLBI
Dr. Elizabeth Nabel, Scientific Director, CRP, DIR, NHLBI

10:15-11:30 **Keynote Speaker**
Chair: Dr. Emily Rothstein
Bruce Alberts, Ph.D., President, US National Academy of Sciences

11:30 - 1:00 **Career Development Panel I - Federal Room**
Chairs: Dr. Kevin Welch
Scientific Journalism
Susan Okie, M.D., Science and Medicine Editor, Washington Post
Pharmaceutical Research
David Christ, Ph.D., Adolor Corporation
Academics
Xandra Breakefield, Ph.D., Professor, Harvard Medical School

1:00 - 2:00 **Roundtable Lunch with Career Development I Panelists**
Scientific Journalism - East Room
Pharmaceutical Research - Jefferson Room
Academics - Federal Room

2:00 - 3:30 **Career Development Panel II** - **Federal Room**
Chairs: Dr. Cheryl Hawkins
Business Development
Sandra G. McElligott, Ph.D., Bio.enterprise LLC.
NIH Extramural
Christine Kelley, Ph.D., Director, Division of Discovery Science and Technology, NIBIB
FDA/Government Research
Ellis Unger, M.D., Center for Biologics Evaluation and Research, FDA

3:30 - 4:30 **Roundtable Discussion with Career Development II Panelists**
Business Development - Federal Room
NIH Extramural - Jefferson Room
FDA/Government Research - East Room

4:30 - 6:30 **Fellows Poster Session and Reception** - Meadow and Studio Rooms
Exhibits of NHLBI Core Facilities

7:00 - 8:00 **Dinner** - Dining Room

8:00 **Awards Presentation** - Federal Room
Drs. Fraser Brown and Paola Pozzi
NHLBI Fellows Award for Outstanding Research
NHLBI Fellows Award for Research Mentoring
Lenfant Fellowship Awards - Dr. Julie Donaldson

8:30 - 11:00 **Informal Social Time/Networking** - Federal Room
Band of Blue (Leader: Ted Mills, Fellow, CB)

June 25

8:00 - 9:00 **Breakfast** - Dining Room

9:00 - 10:00 **Research Highlights by Departing NHLBI Fellows** - I Federal Room
Chair: Dr. Mihaly Kovacs
Qize Wei, Ph.D., LNC and Kansas State University
Shyama Masilamani, Ph.D, LKEM and Virginia Commonwealth University

10:00 - 11:00 **Scientific Speaker**
Chairman, Chair: Dr. Ivan Rosas
Andrew Marks, M.D., Department of Physiology and Cellular Biophysics, Columbia University; Editor, Journal of Clinical Investigation: *Translational Research in Cardiovascular Disease*

11:00 - 11:20 **Break**

11:20 - 11:50 **Research Highlights by Departing NHLBI Fellows - II**
Chair: Dr. Heather Jones
Edward Mills, Ph.D., CB and University of Texas at Austin

12:00 - 2:00 **Lunch/Softball Game** - Pavillion

2:00 - 4:00 **Panel Discussion/Workshops**
Session A: 2:00 - 3:00
Session B: 3:00 - 4:00

Science In China - East Room
Wangquan Zheng, Education Office, Embassy of China

Immigration Issues/Visa Issues - Meadow
Adam Frank and Edward Leavy, Immigration Attorneys, Leavy and Frank, L.L.P.

Family Issues: Managing the Two Career Couple - Pavillion
NHLBI Couples

Becoming an Assistant Professor - Federal Room
Elizabeth Powell, Ph.D., University of Maryland Medical School
NHLBI Departing Fellows

Getting in the Door in Biotech - Pavillion
Dana C. Hilt, M.D., Chief Medical Officer and Senior Vice President, Drug Development, ASCEND Therapeutics

4:15 Departure

Speakers Biographies

Bruce Alberts, Ph.D. is president of the National Academy of Sciences and chair of the National Research Council, which with the National Academy of Engineering and the Institute of Medicine form the National Academies. A respected biochemist recognized for his work in biochemistry and molecular biology, Alberts is known for his extensive molecular analyses of the protein complexes that allow chromosomes to be replicated. Born in Chicago, Dr. Alberts graduated from Harvard College with a degree in biochemical sciences and earned a doctorate from Harvard University in 1965. He joined the faculty of Princeton University in 1966, moving to the medical school of the University of California, San Francisco in 1976. In 1980, he was awarded an American Cancer Society lifetime research professorship. In 1985, he was named chair of UCSF's department of biochemistry and biophysics. He was elected NAS president in 1993. Alberts is one of the original authors of The Molecular Biology of the Cell, now in its fourth edition. His most recent text, Essential Cell Biology, now in its second edition, is intended to present this subject matter to a wider audience. Alberts is committed to improving science education at all levels. He helped to create City Science, a program for improving science teaching in San Francisco elementary schools. At the National Academies, he formed the Center for Education to provide strong support for an evidence-based, continuously improving system of public education in the United States. As NAS president, Alberts has focused on promoting science-based decisionmaking throughout the world. Currently, he is co-chair of the InterAcademy Council, a new organization governed by the presidents of science academies from 15 nations, designed to provide science advice to the United Nations and other international organizations.

Andrew R. Marks, M.D. is Chairman and Professor of Physiology and Cellular Biophysics; Clyde and Helen Wu Professor of Medicine (Molecular Cardiology); Director, Center for Molecular Cardiology; Editor-in-Chief, The Journal of Clinical Investigation. He received his M.D. from Harvard Medical School in 1980, and did his residency in Internal Medicine and his Cardiology Fellowship at Massachusetts General Hospital. A major focus of the laboratory is the characterization of the single channel properties of the cloned expressed ryanodine receptor (RyR)/calcium release channel of the sarcoplasmic reticulum that controls excitation-contraction (EC) coupling in cardiac and skeletal muscle. Our laboratory reported the physical association between FKBP12 and the ryanodine receptor, and showed that FKBP12 (the cytosolic receptor for the immunosuppressant drugs FK506 and rapamycin which also has cis-trans peptidyl-prolyl isomerase activity) modulates the gating of the channel. Our laboratory has described the abnormal regulation of calcium channel expression during human heart failure showing that the cardiac ryanodine receptor is PKA hyperphsphorylated in failing hearts. The PKA hyperphosphorylation causes the ryanodine receptor/calcium release channel to become leaky to calcium resulting in depletion of calcium in the sarcoplasmic reticulum. This defect contributes to impaired contractility in the failing heart.

Moreover, we have shown that the ryanodine receptor is a macromolecular signaling complex that includes kinases (eg. PKA) and phosphatases (PP1 and PP2A) that are bound directly to the channel via adaptor proteins. Furthermore, we have identified highly conserved sequences that bind the kinase and phosphatase adaptor proteins. These binding motifs are present on many ion channels and provide a means of identifying kinase and phosphatase binding sites in multiple signaling systems

Susan Okie, M.D., Science and Medicine Editor, Washington Post. Dr. Okie studied biology at Harvard University, attended Harvard Medical School, and completed her family practice residency at the University of Connecticut, where she also taught interviewing to medical students. In addition to her work as a reporter, Susan Okie is a physician trained in family practice. Although board-certified in family practice, she is not currently practicing medicine. During her early years at The Post, she worked in a local emergency room one day a week. Her recent stories have focused on the science and ethics of cutting-edge treatments (such as fetal surgery, pancreatic islet transplants for diabetics, and hand or limb transplants) as well as public health problems like the rise of drug-resistant tuberculosis and the hormonal treatment of menopause. Her interests include medical ethics, inequities in medicine and health care, the ecology and politics of infectious disease, and interrelations between behavior, social environment, public policy, and illness. Okie first came to the Post as a medical student and has covered medicine and health care on the local and national staffs as well as serving as the paper's science editor. She also spent three years in western Kenya, writing for The Post on the topics of AIDS, family planning, wildlife biology, and African archaeology as well as contributing to two award-winning series by The Post's foreign staff. She has won journalism prizes for her stories on TB and on the DC General Hospital. She is the author of a children's book with astronaut Sally Ride and of a novel (still unpublished)

David Christ, Ph.D. earned his B.S. in Pharmacy from University of Maryland School of Pharmacy and his M.S. and his Ph.D. in Pharmacology from the Medical University of South Carolina. He is now vice president, preclinical development, of Adolor Corporation responsible for overseeing the departments of drug metabolism and pharmacokinetics, process chemistry, safety assessment, and pharmaceutical technologies. Dr. Christ was previously senior director, drug metabolism and pharmacokinetics at Adolor Corporation. Prior to joining Adolor, Dr. Christ was director, research support, metabolism and pharmacokinetics at Bristol Myers Squibb Co., and previously he held several successive positions over a 13-year period with Dupont Pharmaceuticals. Dr. Christ joined Dupont in 1988 as a senior research scientist in the preclinical ADME group, established the company's biotransformation group in 1992 and from 1996-2001 rose to the position of senior director research support. Dr. Christ has authored or co-authored the preclinical sections of several IND and NDA submissions. He currently serves on the Editorial Board, Drug Metabolism and Disposition and previously served on the Executive Committee, Drug Metabolism subsection of ASPET.

Xandra Breakefield, Ph.D. graduated from Wilson College in 1965 and received her PhD in Microbial Genetics from Georgetown University. She was a postdoctoral fellow with Marshall Nirenberg at the NIH. She was appointed an Assistant Professor in the Department of Human Genetics at Yale Medical School in 1974, and moved in 1984 to Harvard Medical School and Massachusetts General Hospital. She is currently Professor of Neurology at Harvard Medical School. Dr. Breakefield is a basic scientist with a strong background in molecular genetics and neuroscience, who has focused her efforts on generation of novel vectors for gene delivery to neurons and glioblastomas. She has played a pivotal role in the development of herpes virus vectors for gene delivery to neurons, including both herpes simplex virus type 1 (HSV) recombinant virus and amplicon vectors. She and her colleagues, Robert Martuza and Antonio Chiocca, extended use of HSV vectors to gene therapy for brain tumors, and were the first to demonstrate selective killing and on-site vector propagation using replication-conditional HSV vectors. She has organized and taught a number of courses on gene therapy. She directed a Cold Spring Harbor course for nine years, and has directed a gene therapy course at Harvard Medical School for the past two years. Together with Dr. Chiocca she edited, "Gene Therapy for Neurological Disorders and Brain Tumors." Her administrative responsibilities have included the directorships of the Division of Molecular Neurogenetics at the Eunice Kennedy Shriver Center, and the Neuroscience Gene Therapy Program at Massachusetts General Hospital. She is an author of over 250 publications. She serves on the editorial boards of Human Gene Therapy, Gene Therapy, Brain Research, Cancer Gene Therapy and Neurogenetics. She has also served on several NIH Study Sections, on the NINDS Board of Scientific Counselors, on the Dana Awards Nominating Committee, and on the National Gene Vector Laboratories Scientific Review Board. Dr. Breakefield has received a number of awards for her work, including a MacKnight Foundation Neuroscience Development Award, a Javits Neuroscience Investigator Award, the Matilde Soloway Award in Neuroscience, and an honorary doctoral degree from Wilson College.

Sandra Fitzpatrick McElligott, Ph.D. received her Ph.D. in Biology from Temple University and her post-doctoral training in molecular neuroscience at the University of Pennsylvania and University of California at Berkeley. She is now the President, CEO and Co-founder of Bio.enterprise LLC., whose mission is to assist emerging and growing life science companies to gain lasting success. Through our expertise in funding strategies, business development, product development and intellectual property expansion, we help our clients to achieve and surpass their objectives. Dr. McElligott has over 20 years of experience in life sciences and biotechnology with both Fortune 50 companies and small startup companies raising several millions in grants and funding. As Executive Vice President of research at a start-up medical device company, Dr. McElligott raised financing and developed an ultrasound medical device for non-invasive delivery of insulin and other proteins. While the Vice President of Research at a DuPont ConAgra joint ventures, Dr. McElligott developed several products for the human and animal health-care markets based on antibody

and anti-inflammatory nutritional components. Previously, she was a senior scientist in the Central Research & Medical Products Division of DuPont, conceptualizing and developing products for the molecular biology markets. Dr. McElligott holds four issued US patents and nine pending patent applications.

Dr. Christine A. Kelley is the Director of the Division of Discovery Science and Technology at the NIBIB. She received her Ph.D. degree in Cell Biology from Boston University in 1988. Her graduate research focused on the role of pericytes in the microvasculature. From 1988-1996 Dr. Kelley conducted postdoctoral and independent research on the function and regulation of smooth muscle and nonmuscle myosin isoforms in the Laboratory of Molecular Cardiology in the National Heart, Lung, and Blood Institute (NHLBI). In 1996 Dr. Kelley became a Health Scientist Administrator in the Vascular Biology Research Group within the Division of Heart and Vascular Diseases in the NHLBI, before moving in 1998 to a position as a Health Scientist Administrator in the Bioengineering and Genomic Applications Research Group within the same Division. Dr. Kelley assumed her current position in NIBIB in March, 2002.

Dr . Ellis F. Unger received his M.D. in 1980 from the University of Cincinnati College of Medicine. He then did a residency in Internal Medicine at the Medical College of Virginia. He then came to NHLBI where he first was as Medical Staff Fellow, and from 1988 to 1997 was a Senior Investigator, Cardiology Branch. In 1997, Dr. Unger moved to the FDA as Medical Officer and now holds the position of Acting Chief, General Medicine Branch, Division of Clinical Trial Design and Analysis, Office of Therapeutics Research and Review, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration. While at NIH, he sought to develop angiogenesis as a therapeutic modality for ischemic cardiovascular disease. This involved many years of laboratory work (1985-1994), and culminated in a clinical protocol for patients with coronary artery disease. He was the first US clinical investigator to administer angiogenic growth factors parenterally to patients with cardiovascular disease. This body of work also led to a DHHS patent.

Mr. Wangquan Zheng is the Second Secretary for the Embassy of the People's Republic of China in Washington, D.C. He majored in Comparative Education at the Beijing Normal University. His thesis was entitled “The Experience of Undergraduate Education in America”. As a major part of higher education, undergraduate education plays an important role in the fostering of mid-&-advanced talents. This dissertation focussed on research and analysis of advantages and disadvantages of undergraduate education in the United States, in order to develop suggestions and proposals on the development of undergraduate education in China.

Adam Frank, Immigration Attorney, Leavy and Frank, L.L.C. received his B.A. in Political Science from Brandeis University in 1990, and then went to the University of Baltimore School of Law, where he received his J.D. in 1994. His practice areas include criminal law, family law, labor law and immigration law. He is a member of the Maryland Bar Association and the National Lawyers Guild. He has lectured on the topic of "Hiring Foreign Professionals".

Edward N. Leavy, Immigration Attorney, Leavy and Frank, L.L.C. received his B.A. in 1964 from Columbia University, and a J.D. from Brooklyn Law School in 1968 and an LL.M. from New York University in 1974. His practice areas include criminal law, employment law, family law, labor law and immigration law. He is a member of the American Immigration Lawyers Association.

Elizabeth Powell, Ph.D., University of Maryland Medical School was trained in Biomedical Engineering as an undergraduate at The Johns Hopkins University. Following a Masters in Chemical Engineering designing polymer substrates for neural regeneration, she pursued an interdisciplinary doctoral program in Chemical Engineering and Developmental Biology at Rutgers with Dr. Herbert Geller and did postdoctoral training with Dr. Pat Levitt at the University of Pittsburgh. She found that the family of plasminogen activators, classically responsible for dissolving blood clots, and the plasminogen related growth factors (including hepatocyte growth factor/scatter factor (HGF/SF)) are critical components of interneuron development. In transgenic mutant mice, the lack of these molecules results in interneuron loss and behaviors similar to human epilepsy, autism and anxiety disorders. As an Assistant Professor since 2003, her lab is directed at studies of the molecular and cellular mechanisms of forebrain GABAergic interneurons: What specifies their cell fate? How do they migrate to their final destination? And what factors regulate interneuron differentiation and circuit formation? The laboratory uses a wide variety of approaches from the molecular level to animal behavioral testing to explore how developmental alterations lead to adult neurological and psychiatric disorders. Our long-term goals include designing interventions to prevent or ameliorate these conditions.

Dana C. Hilt, M.D. is the Chief Medical Officer and Senior Vice President of Drug Development, ASCEND Therapeutice. Dr. Hilt received his medical degree from Tufts University School of Medicine and his Bachelor of Science degree from the University of Maine. He is responsible for research and clinical development programs. Dr. Hilt has industry experience in developing both pharmaceutical and biologic human therapeutics. Most recently, he held the title of Vice President of Clinical Research, Drug Metabolism and Toxicology and Chief Medical Officer of Guilford Pharmaceuticals. In this capacity, Dr. Hilt directed preclinical and clinical development efforts, as well as interactions with the FDA and other international regulatory agencies. Prior to joining Guilford, Dr. Hilt served as Director of Clinical Neuroscience at Amgen. In this capacity, he oversaw development of the company's

pipeline of neurotrophic factors for several CNS indications, including GDNF (glial cell-line-derived neurotrophic factor), BDNF (brain-derived neurotrophic factor) and NT-3 (neurotrophin 3). In addition to Dr. Hilt's industry experience, he has had a prominent academic career as Associate Professor of Neurology at several schools of medicine, including the University of Maryland, the University of Southern California and the Baltimore Veteran's Medical Center.

NHLBI Core Facilities

Bioinformatics Core Facility

Eric Billings, Ph.D., Director
Building 10, Room 4A15, E-mail: billingse@nhlbi.nih.gov
Phone: (301) 496-6520, Fax: (301) 480-1243

The NHLBI Bioinformatics Core Facility is chartered to facilitate intramural scientists' research by providing access to existing and new tools for both discovery and hypothesis driven research. The core will support advanced users with application software; provide training for new and advanced users; facilitate access to Affymetrix and new spotted array scanners; field general questions about Bioinformatics tools; provide collaborative research support with post-doctoral bioinformatics staff; provide easy access to in-house workstations loaded with bioinformatics research tools. In the long term, nn integrated approach to genomics and proteomics is being developed within the Core Facility. Academic, commercial and open source approaches are being combined to provide a platform for addressing scientific questions that span genomic, proteomic and other information sources.

Electron Microscopy Core Facility

Zu-Xi Yu, M.D., Ph.D., Facility Manager:
Building 10, Room 2N246, E-mail: yuz@helix.nih.gov
Phone: (301) 402-0908, Fax: (301) 402-4127

The Core Electron Microscopy Facility at NHLBI provides contemporary transmission electron optical services to all investigators within the Institute. Services include standard light microscopy, transmission electron microscopy of cells and tissues, immuno-electron microscopy (immunocytochemistry at the electron microscopic level, including immuno-gold labeling) on ultrathin plastic or frozen sections, negative staining or rotary shadowing of macromolecule preparations (nucleic acids or proteins), autoradiography at the light and electron microscopic level, computer-assisted morphometry and image analysis, diagnostic pathology at both histological and ultrastructural level, and full range of traditional dark room services.

Flow Cytometry Core Facility

J. Philip McCoy, Jr., Ph.D., Director
Building 10, Rooms 4A07 and 4A11, E-mail: mccoyj@nhlbi.nih.gov
Phone: 301-594-6950, Fax: (301) 480-4774

The mission of the NHLBI Flow Cytometry Core Facility is to provide investigators at the NHLBI access to state-of-the-art flow cytometry. This is done by having cytometers and software available in the core facility and by providing consultation to investigators who have cytometers available in their own laboratories or branches. Investigators are responsible for specimen preparation and staining.

The staff of the flow cytometry laboratory will gladly assist you in designing your experiments and in developing optimal preparation and staining procedures. For analytical experiments, data will be provided as either hard copies or on appropriate media as listmode files. Summit software (Cytomation, Inc) will be available for "offline" analysis of these files. For sorting experiments, each investigator is responsible for bringing appropriate media and test tubes.

Light Microscopy Core Facility

Christian A. Combs, Ph.D., Facility Director
Building 10, Room 5D19; E-mail: combsc@nhlbi.nih.gov
Pager: (301) 930-3020

The mission of the light microscopy core facility is to provide state of the art equipment, training, and image processing capabilities to assist researchers within the NHLBI-DIR in experiments involving light microscopy. Equipment within the facility includes several types of confocal microscopes, a two-photon microscope, and a standard epi-fluorescence widefield microscope. This range of instruments provides capabilities that include live cell imaging, deep tissue-level imaging, video-rate confocal imaging, spectral imaging, and simple widefield fluorescence and brightfield imaging of prepared slides Image processing capabilities include deconvolution, digital linear unmixing of spectrally overlapping fluorochromes, and 3D reconstruction as well as a custom in-house image processing programs for specific applications. It is intended that this webpage provide researchers with all of the information necessary to plan their experiments based on the capabilities of the core instruments as well as to provide background information on the light microscopy techniques that are available.

Pathology Core Facility

Zu-Xi Yu, M.D., Ph.D., Facility Head
Building 10, Room 2N240; E-mail: yuz@helix.nih.gov
Phone: (301) 402-0908, Fax: (301) 402-4127

The Pathology Core is a Morphology Core Facility, which provides histopathological, immunocytochemical, and ultrastructural support for NHLBI intramural research. The Core Facility provides quality control for morphologic studies, experimental pathology (animal models) and optimizes use of supplies and equipment for all investigators at the NHLBI in which morphological studies and tissue-based molecular studies play a critical role. Ongoing interaction of Pathology Core personnel with each investigator facilitates communication regarding morphologic findings, histopathological interpretation, and new technical developments, thus increasing the efficiency of the research projects. Staff are well-trained, extremely experienced technicians, and the laboratory has a wide repertoire of specialized techniques. The research pathology and immunohistochemistry are subsequently operating using standard operating procedures based on good lab practice guidelines.

Proteomics Core Facility
Rong-Fong Shen, Ph.D., Chief
Bldg. 10, Room 6C208; E-mail: ShenR@nhlbi.nih.gov
Phone: (301) 594-1060, Fax: (301) 402-2113

The mission of the Core is to facilitate investigators in the NHLBI's Division of Intramural Research accomplish their proteomics endeavors by providing mass spectrometry-based analyses of samples. The Core maintains state-of-the-art mass spectrometers, provides guidance to sample preparation, trains scientists and research fellows for the use of instrument and data analysis, and develops analytical methodologies relevant to proteomics.

Transgenic Mouse Core Facility
Chengyu Liu, Ph.D., Chief
Building 50, Room 3537; E-mail: Liuch@nhlbi.nih.gov
Telephone: (301) 435-5034, Fax: (301) 435-4819

The NHLBI Transgenic Facility is a state-of-the-art transgenic mouse laboratory established in 1999. Its main function is to assist NHLBI scientists making transgenic and knockout mice. Generally, the users of the facility are responsible for making the DNA constructs, and screening the potential positive ES cell clones and founder mice. The staff at the facility are responsible for the culturing, transfecting, and selecting ES cells, as well as microinjecting and implanting mouse embryos. After creation, the transgenic or knockout mice are transferred to each users' animal room for phenotypic analysis. We use the standard pronuclear microinjection method to make transgenic mice. The users are responsible for making the DNA constructs and purifying the fragments for microinjection. Generally, the transgene needs to be separated from the cloning vector and carefully purified before submitting to our facility. Our standard strain of mice is B6CBAF1/J (C57BL/6J x CBA/J). We can also make transgenic mice using FVB/NJ and C57BL/6J inbred strains if necessary. For the production of knockout mice, the users are responsible for making the targeting constructs and providing us with at least 100 microgram of linearized, highly intact DNA (freshly prepared and digested, or store in 70% ethanol at -20˚C). The DNA samples will be electroporated into embryonic stem (ES) cells. The correctly-targeted ES cell clones will be microinjected into blastocyst stage embryos (blastocyst microinjection) to create chimeric mice. At appropriate age, the visibly good chimeric mice will be mated with C57BL and 129 mice to pass the mutated gene to the next generation. Our facility is equipped with microscopes and manipulators that are suitable for dissecting and micro-manipulating mouse embryos and cultured cells. We are also interested in in vitro differentiation of the ES cells through the formation of embryoid bodies (EB).

Poster Session Titles and Assignments

Posters are put up in the morning

Authors of odd numbered posters present 4:30-5:30

Authors of even numbered posters present 5:30-6:30

Biochemistry & Signal Transduction

1. **Arf6 regulates Rac1-induced ruffling by interacting with human kalirin in Hela.** T.H. Koo and J.G. Donaldson; Laboratory of Cell Biology.

2. **ATM is Activated by High NaCl, Contributing to Increased TonEBP/OREBP-mediated Transcription.** C.E. Irarrazabal, M.B. Burg, and J.D. Ferraris; Laboratory Kidney Electrolyte Metabolism.

3. **Cardiac-Specific Homeodomain NKx-2.5: Conformational Stability and Specific DNA Binding.** E. Fodor[1], J.W. Mack[2], J. A. Ferretti[2], and A. Ginsburg[1]; [1]Laboratory of Biochemistry, [2]Laboratory of Biophysical Chemistry.

4. **Characterization of the Ezymatic Function of Nonmuscle Heavy Meromyosin II-B and II-C.** K. Y. Kim, Y. A. Preston, M. Kovacs, E. V. Harvey, J. R. Sellers, and R. S. Adelstein; Laboratory of Molecular Cardiology.

5. **Identification of Human Methyl-CpG Binding Domain Protein (MBD) 4 as a Substrate of Protein Kinase X**. W. Li, R. M. Kotin; Lab of Biochemical Genetics.

6. **Identification of Promoter Elements in Mouse PDE3B Gene: The Role of CREB in PDE3B Expression**. H. Liu, J. Tang, V. C. Manganiello; Pulmonary Critical Care Medicine Branch.

7. **Identification of NEFA as an ARTS-1 Interacting Protein that Promotes the Release of Soluble TNFR1.** A. Islam, S. J. Levine; Pulmonary Critical Care Medicine Branch.

8. **Inhibition of Apoptosis in Acute Promyelocytic Leukemia Cells Leads to Increase in Levels of Oxidized Protein and LMP2 Immunoproteasome.** M. A. S. Khan, H. Oubrahim, and E. R. Stadtman; Laboratory of Biochemistry.

9. **Mechanistic Insights Revealed Through Characterization of a Novel Chromophore in Selenophosphate Synthetase from E. coli. M. D. Wolfe, T. C. Stadtman; Laboratory of Biochemistry.**

10. **Myosin V Moves by a Hand-over-Hand Lever Arm Mechanism.** T. Sakamoto[1], A. Yildiz[2], P. R. Selvin[2], J. R. Sellers[1]; [1]Laboratory of Molecular Cardiology, [2] Physics Dept. & Biophysics.

11. **Proteomic analysis of calcium-induced phosphorylation events in porcine heart mitochondria.** R.K. Hopper, S. Carroll, and R.S. Balaban; Laboratory of Cardiac Energetics.

12. **Sumoylation of Heterogeneous Nuclear Ribonucleoproteins, Zinc Finger Proteins, and Novel Nuclear Pore Complex Proteins.** T. Li, P. B. Chock; Laboratory of Biochemistry.

13. **The DNA Binding Agents Hoechst 33258 and 33342 Enhance Recombinant Adeno-Associated Virus (rAAV) Transgene Expression**. L. Li, L. Yang, R. M. Kotin; Laboratory of Biochemistry Genetics.

14. **Unique Mechanism of Action of Myosin-X, a Membrane Motor.** M. Kovacs, F. Wang, J. R. Sellers; Laboratory of Molecular Cardiology.

Cell Biology

15. **Association of Osmotic and Oxidative Stress.** Z. Zhang[1], N. I. Dmitrieva[1], J. H. Park[2], R. L. Levine[3], and M. B. Burg[1]; [1] Laboratory of Kidney and Electrolyte Metabolism, [2] Laboratory of Biochemistry, [3] Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research.

16. **Exchange of Clathrin on Clathrin-Coated Pits in Permeabilized Tissue Culture Cells.** Y.I. Yim, S. Scarselletta, F. Zang, D.-W. Lee, L. Greene and E. Eisenberg; Laboratory of Cell Biology.

17. **Intracellular Localization of Myosin18A.** Y. Yang, Q. Xu, and J. Sellers; Cell Motility Group.

18. **Membrin is a Golgi Membrane Receptor for Arf-1.** A. Honda and J. G. Donaldson; Laboratory of Cell Biology.

19. **Nuclear Localization and Molecular Partners of Big1, a Brefeldin A-inhibited Guanine Nucleotide-exchange Protein for Adp-ribosylation Factors.** P. I. Padilla, G. Pacheco-Rodriguez, J. Moss, and M. Vaughan; Pulmonary Critical Care Medicine Branch.

20. **Requirement for Cx43a1 in heart development arises from an essential role in coronary vasculogenesis.** D.L. Walker, M.L. Kirby, C.W. Lo; Laboratory of Developmental Biology.

21. **Rescuing siRNA Induced Defects in Cytokinesis by Myosin II Isoforms**. J. Bao, Q. Wei, S. Jana, R. Adelstein; Laboratory of Molecular Cardiology.

22. **Sulfation of Astrocyte-derived Proteoglycans is Essential for the Control of Neuronal Growth**. H. Wang, Y. Katagiri, H. M. Geller; Developmental Neurobiology Group.

23. **Tissue and Cellular Distribution of an Inserted Isoform of Nonmuscle Myosin II-C.** S. S. Jana, S. Kawamoto, R. S. Adelstein; Laboratory of Molecular Cardiology.

Injury/Inflammation/ Immunology

24. **Activation of Synoviocytes by the Phospholipase Motif of Parvovirus B19 Capsid Protein (VP1):** J. Lu, N. Zhi, and K. E. Brown; Hematology Branch.

25. **Genomic Analysis of the Hepatoprotective Role of *N*-Acetyl-L-Cysteine.** M. L. Adams[1], M. F. Radonovich[2], J. N. Brady[2], L. R. Pohl[1]; [1]Laboratory of Molecular Immunology, [2]Virus Tumor Biology Section, National Cancer Institute.

26. **Inducible Mitochondrial Uncoupling – An Endogenous Regulatory Mechanism Augmenting Tolerance to Cardiac Ischemia.** C. J. McLeod[1], J. P. McCoy Jr[2], R. F. Hoyt Jr[3], M. N. Sack[1] ; [1]Cardiovascular Branch, [2]Flow Cytometry Core, [3]Laboratory of Animal Medicine and Surgery.

27. **RBMX, a RNA Binding Motif Protein, Interacts with Membrane-Associated ARTS-1 and Promotes Soluble TNFR1 Release.** B. Adamik, F. N. Rouhani, F. I. Hawari, and S. J. Levine; Pulmonary Critical Care Medicine Branch.

28. **Reduction of TFII H Complex is Associated with Global Down-regulation of Alveolar Macrophage Transcription and Gene Expression in Idiopathic Pulmonary Fibrosis.** P. Ren, I. Rosas, H.P. Wu, S.D. MacDonald, B.R. Gochuico; Pulmonary Critical Care Medicine Branch.

29. **Release of Full-Length, 55-kDa, TNF Receptor 1 in Exosome-Like Vesicles: A Novel Mechanism for Generation of Soluble Cytokine Receptors.** F. I. Hawari, F. N. Rouhani, X. Cui, Z.-X. Yu, C. Buckley, M. Kaler, and S. J. Levine; Pulmonary Critical Care Medicine Branch.

30. **Role of Cyotkine Networks in Determining Susceptibility to Drug-induced Liver Disease (Dild)**. M. Holt[1], M. Bourdi[1], D. Eiras[1], T. Reilly[2], K. Welch[1], H. Amouzadeh[1], and L. Pohl[1]; [1]Molecular and Cellular Toxicology Section, [2]Bristol-Myers Squibb, Drug Safety Evaluation, Syracuse, NY.

31. **Suppression of Toll-Like Receptor (TLR) Mediated Activation of Mast Cells by Dexamethasone.** H. Qiao and M. A. Beaven; Laboratory of Molecular Immunology.

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38. **Relative Quantification of Highly Complex Proteomes Using Liquid Chromatography and Nano-Spray Tandem Mass Spectrometry.** G. Wang, W. W. Wu, W. Zeng, C.-L. Chou, and R.-F. Shen; Proteomics Core Facility, Hematology Branch, Laboratory of Kidney and Electrolyte Metabolism.

39. **High Throughput Discovery of Mutations in Mouse Connexin Using Resequencing DNA Chip and Wave.** Y. Wu, E. Doe, C. Rosenow*, C. W. Lo; Laboratory of Developmental Biology.

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41. **Genetic and Hormonal Characterization of Meningiomas in Patients with Lymphangioleiomyomatosis (LAM).** D.M. Crooks[1], R. DeCastro[1], J. Valencia[2], C. Glasgow[1], N. Patronas[3], J. Moss[1]; [1]Pulmonary Critical Care Medicine Branch, [2]Pathology Section, and [3]Department of Diagnostic Radiology.

42. **Towards Novel Therapeutics against HIV Infection and Drug Resistance.** Y. Che, R. B. Brooks; Lab of Biophysical Chemistry.

ABSTRACTS

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RBMX, a RNA Binding Motif Protein, Interacts with Membrane-Associated ARTS-1 and Promotes Soluble TNFR1 Release. B. Adamik, F. N. Rouhani, F. I. Hawari, and S. J. Levine; Pulmonary Critical Care Medicine Branch.

We have identified that ARTS-1 (Aminopeptidase Regulator of Type I TNF Receptor Shedding) binds to and promotes the release of soluble TNFR1 from pulmonary epithelial cells. We hypothesized that the mechanism by which ARTS-1 promotes the release of soluble TNFR1 involves other ARTS-1-interacting proteins. Co-immunoprecipitation experiments were performed on membrane fractions from NCI-H292 cells utilizing an anti-ARTS-1 antibody. An ARTS-1-interacting protein was identified by SDS-PAGE, Coomassie blue staining, and MALDI-MS peptide mass mapping as RBMX. To confirm the interaction between ARTS-1 and RBMX, we expressed a His-tagged RBMX protein in COS7 cells and demonstrated that ARTS-1 was co-immunoprecipitated with an anti-His antibody. Although RBMX belongs to the family of heterogeneous nuclear ribonucleoproteins, which are involved in pre-mRNA post-transcriptional modifications, we hypothesized that it might participate in the regulation of soluble TNFR1 release via its interaction with ARTS-1. The quantity of TNFR1 in the medium from an anti-sense RBMX COS7 cell line was significantly reduced as compared to mock-transfected cells (mean 226.8 ± 7.4 pg/ml vs. 760.4 ± 43.8 pg/ml, n=6, $p < 0.00001$). Immunoblots of membrane fractions revealed that the anti-sense RMBX cell line contained more TNFR1 than mock-transfected cells, consistent with impaired soluble TNFR1 release. Similarly, the quantity of TNFR1 in the medium from the anti-sense RBMX COS7 cell line following stimulation with 10^{-7} M PMA for 24 hrs was significantly reduced as compared to PMA-stimulated mock-transfected cells (442.5 ± 19 pg/ml vs. 2,607.4 ± 196 pg/ml, $n = 6$, $p < 0.001$). In conclusion, we have identified RBMX as a novel ARTS-1-interacting protein that plays an important role in promoting both constitutive and inducible release of soluble TNFR1.

Genomic Analysis of the Hepatoprotective Role of *N*-Acetyl-L-Cysteine. M. L. Adams[1], M. F. Radonovich[2], J. N. Brady[2], L. R. Pohl[1]; [1]Laboratory of Molecular Immunology, [2]Virus Tumor Biology Section, National Cancer Institute.

N-acetyl-L-cysteine (NAC) is a well established therapeutic agent commonly used as a mucolytic as well as an antidote for acetaminophen-induced hepatotoxicity. More recently, NAC has been investigated as treatment for other forms of hepatic failure or prophylaxis for ischemia-reperfusion injury and radiocontrast media-induced nephropathy. Although the protective effects of NAC are believed to be mediated in part by its conversion into glutathione, the molecular basis of the pharmacological activity of NAC remains unclear. In an effort to help resolve this problem, the genome-wide changes in hepatic mRNA expression of wild type C57Bl/6J mice 3h after treatment with NAC (450 mg/kg, i.p.) was investigated with the use of Affymetrix Murine Genome U74v2 Set GeneChip® Array. The results were compared to the expression data obtained following PBS vehicle treatment. Using the microarray analysis program GeneSpring, a gene list of 1,594 genes with either at least two fold increase (1,103 genes) or decrease (491 genes) in expression level was generated. Several gene categories of interest, including cell cycle regulation, acute phase response, cell adhesion molecules, chaperone

proteins, and growth factors were effected by NAC treatment. Additionally, changes were observed in glutamate-cysteine ligase modifier subunit, caspase-I, glutathione peroxidase-3, hepatocyte growth factor, as well as metallothioneins 1, and 2. These findings suggest that the protective effects of NAC are likely due to its modulation of multiple networks involved in cellular homeostasis.

Rescuing siRNA Induced Defects in Cytokinesis by Myosin II Isoforms. J. Bao, Q. Wei, S. Jana, R. Adelstein; Laboratory of Molecular Cardiology.

Here we report on RNAi treatment of monkey COS-7 cells, a cell line that lacks nonmuscle myosin heavy chain (NMHC) II-A, but contains NMHC II-B as well as II-C. We made use of COS-7 cells to investigate the role of the different NMHC isoforms in cytokinesis. We used 21 nucleotide small interfering RNA duplexes (siRNA) to specifically suppress expression NMHC II-B. Quamtitative immunoblot analysis showed that compared to control cells, NMHC II-B expression levels decreased to 16.0±0. 8% after 72 hours and 10.2±0.7% after 96 hours. There was no alteration in the expression level of NMHC II-A and II-C. Down-regulation of NMHC II-B expression significantly inhibited cell proliferation so that cell numbers were approximately 50% lower in the RNAi treated cells compared to control cells. Whereas 8.7±1.0% of control cells were multi-nucleated, 62.4 ± 8.8% of the RNAi treated cells became multi-nucleated 72 hours after transfection. Moreover, RNAi-treated cells had increased surface areas and, unlike control cells, lacked actin stress fibers. We assayed the ability of the various NMHC II isoforms to rescue multinucleation by introducing GFP-tagged NMHC II isoforms into II-B siRNA treated cells. We found that the multinucleation in these cells was reduced from 65.8% ± 5.7% to 17.8 ± 2.2% by GFP-NMHC II-B. Interestingly, GFP-NMHC II-A reduced the percent of multinucleated cells to 29.8±7.4%, but GFP-NMHC II-C could not rescue multinucleation, 62.2±7.1% of the COS-7 cells remained multinucleated (n=4 for all experiments). Similar to NMHC II-B, II-A localized to stress fibers partially overlapping actin filaments, whereas II-C was more punctate in its appearance and showed little overlap with actin filaments. These studies show that NMHC II-C alone is insufficient for normal cytokinesis and also suggest that NMHC IIA can, at least partially, rescue the defect in cytokinesis due to the loss of NMHC IIB.

Towards Novel Therapeutics against HIV Infection and Drug Resistance. Y. Che, R. B. Brooks; Lab of Biophysical Chemistry.

The efficacy of antiretroviral agents approved for the treatment of HIV-1 infection is limited by the virus's ability to develop resistance. As such there is an urgent need for new ways of thinking about anti-HIV drug development, and accordingly novel viral and cellular targets critical to HIV-1 replication need to be explored and exploited. Many proteins exert their biological roles as components of complexes, and the functions of proteins are often determined by their specific interactions with other proteins, suggesting the modulation of protein-protein interactions provide alternative targets for HIV-1 inhibition. Two structure-based methods are being developed to discover small molecules capable of modulating specific protein interactions: (a) Mimicry of important protein-recognition motifs, such as ?-helix, ?-sheet, turn and loop structures, with appropriate side-chains attached,

to disrupt protein-protein interaction directly; (b) Docking small molecules to protein cavities distal from complex interfaces and to allosterically modulate protein interactions. Based on these approaches, we have developed novel lead compounds against HIV infection, including small molecules inhibiting viral attachment to chemokine receptor CCR5/CXCR4, membrane fusion inhibitors arresting pre-hairpin complex of gp41, non-nucleoside reverse-transcriptase inhibitors, dimerization inhibitors of HIV protease, oligomerization inhibitors of HIV integrase and maturation inhibitors of HIV capsid proteins.

Genetic and Hormonal Characterization of Meningiomas in Patients with Lymphangioleiomyomatosis (LAM). D.M. Crooks[1], R. DeCastro[1], J. Valencia[2], C. Glasgow[1], N. Patronas[3], J. Moss[1]; [1]Pulmonary Critical Care Medicine Branch, [2]Pathology Section, and [3]Department of Diagnostic Radiology.

LAM is a cystic lung disease of women that is associated with abdominal and thoracic tumors (e.g., angiomyolipomas, lymphangioleiomyomas). It is believed to be caused by a neoplastic "LAM" cell having a smooth muscle-like phenotype, and may occur as sporadic disease, or in association with tuberous sclerosis complex (TSC), an inherited disorder. In a previous study, we reported that the incidence of meningiomas was higher in LAM than in the general population. These initial findings have now been extended through evaluation of additional patients, as well as by genetic analysis. Sixteen of 360 patients with LAM or LAM/TSC were shown by MRI/CT to have meningiomas. Of these, three required surgery for probable meningioma-induced symptoms. This number significantly exceeds the frequency of meningioma (~1%) found at autopsy in the general population. The tumors found in LAM patients are typical of benign grade I meningiomas by both radiological and histological criteria. DNA extracted from microdissected meningioma tissue of a sporadic, LAM patient was screened for loss of heterozygosity (LOH) of polymorphic microsatellite markers. No LOH was detected near the *TSC1* or *TSC2* genes, nor at chromosome arms 14q and 1p, a characteristic of grade II and grade III tumors. Allelic imbalances were identified at markers in proximity to *NF2* (neurofibromatosis 2), a locus at which LOH is detected at a frequency of 50-70% in meningioma. Meningioma cells are sensitive to growth factors, showing increased growth in response to platelet-derived growth factor, insulin-like growth factor and progesterone. LAM cells have been shown to produce growth factors, and some LAM patients are treated with progesterone. These results indicate that hormonal as well as genetic factors may play a role in the predisposition to meningioma in LAM patients. Funded by: NHLBI Division of Intramural Research.

Cardiac-Specific Homeodomain NKx-2.5: Conformational Stability and Specific DNA Binding. E. Fodor[1], J.W. Mack[2], J. A. Ferretti[2], and A. Ginsburg[1]; [1]Laboratory of Biochemistry, [2]Laboratory of Biophysical Chemistry.

The NKX-2.5 homeobox gene is expressed at different stages of the cardiac development, regulating atrial septation and left ventricle formation. Mutations in the homeodomain itself cause various congenital hearth disease. The mouse NKx-2.5 homeodomain protein has 99% sequence identity with the human NKX-2.5 homeodomain and 73% identity, and similar NMR structure with the parent member

of the NK-2 family, the vnd (ventral nervous system defective)/NK-2 homeodomain of Drosophila melanogaster studied earlier. Here we studied the NKx-2.5 homeodomain where an oxidizable Cys at position 56 has been replaced by Ser to produce NKx-2.5(C56S) and was expressed as a 79-residue protein. The thermal stability of NKx-2.5(C56S) has been investigated by differential scanning calorimetry (DSC) and far-UV circular dichroism (CD). Consecutive scans at 60°C/h from 10 to 80 °C give a single, reproducible transition with DH = 34±2 kcal mol-1 for NKx-2.5(C56S) at both pH 6.0 or 7.4 in the absence of NaCl. Using either DSC or CD, the data are fitted well to a two-state unfolding model with unit cooperativity. The addition of NaCl increases protein stability at both pH values. Addition of 200 mM NaCl at pH 7.4 increases the midpoint of the transition of NKx-2.5 from 44.2 to 56.4 °C and gives a DCp value for unfolding of 0.85 kcal K-1 mol-1. The binding of the NKx-2.5 homeodomain to duplex 18 bp DNA containing the specific 5'-CAAGTG-3' binding sequence as its core was studied at pH 7.4 in the presence of 100 mM NaCl by isothermal titration calorimetry. Titrations of the 18 bp DNA with NKx-2.5(C56S) at different temperatures yield a binding constant of 108 M-1. From 10 to 40 °C, the enthalpy of binding decreased linearly giving an estimated value for DCp of -180±10 cal K -1 mol-1 for the homeodomain binding specific duplex DNA.

Release of Full-Length, 55-kDa, TNF Receptor 1 in Exosome-Like Vesicles: A Novel Mechanism for Generation of Soluble Cytokine Receptors. F. I. Hawari, F. N. Rouhani, X. Cui, Z.-X. Yu, C. Buckley, M. Kaler, and S. J. Levine; Pulmonary Critical Care Medicine Branch.

Pulmonary innate immune events can be regulated by soluble cytokine receptors. The goal of this study was to investigate further the mechanism of shedding of the type I, 55-kDa tumor necrosis factor receptor (TNFR1), which has been recognized to be mediated via the proteolytic cleavage of the 28-kDa extracellular domain. We unexpectedly found that the predominant form of soluble TNFR1 present in human serum is the full-length, 55-kDa protein. We hypothesized that soluble TNFR1 might be generated by the release of exosome-like vesicles containing full-length, 55-kDa, membrane-associated TNFR1. Immunoblots of exosome-like vesicles, isolated from medium from human vascular endothelial cells (HUVEC) by sequential, high-speed centrifugation (175,000 x g for 16 hr), demonstrated only full-length, 55-kDa TNFR1. Full-length, 55-kDa TNFR1 in HUVEC medium floated at a specific gravity of 1.1 g/ml on continuous sucrose gradients, consistent with its localization in exosome-like vesicles. Bronchoalveolar lavage fluid (BALF) was also collected from normal volunteers and subjected to sequential, high-speed sequential centrifugation. In 4 of 7 BALF samples, only full-length 55-kDa TNFR1 was sedimented (100,000 x g for 1 hr), consistent with localization of TNFR1 in exosome-like vesicles, while in 3 BALF samples, both the full-length 55-kDa TNFR1 and the cleaved, 28-kDa soluble TNFR1 ectodomain were present. The presence of TNFR1 in exosome-like vesicles from HUVEC medium and BALF was confirmed by electron microscopy with immunogold labeling, which localized TNFR1 to vesicles of 20 nm to 50 nm in diameter. TNFR1 exosome-like vesicles do not possess intrinsic signaling capabilities based upon the presence of SODD (Silencer of Death Domains) and the absence of an active TNFR1 signaling

complex I (TRADD, TRAF2, and RIP). TNFR1 exosome-like vesicles do not contain lipid raft microdomains, as evidenced by their partitioning to Triton X-100 soluble, non-lipid raft fractions on discontinuous sucrose gradients where TNFR1 co-localizes with Fas. Therefore, under basal conditions, exosome-associated TNFR1 is inactive. Further, the broad-spectrum zinc metalloprotease inhibitor, TAPI-2, decreases the release of TNFR1 exosome-like vesicles, consistent with a role for zinc metalloproteases in this process. In summary, we have identified that the release of exosome-like vesicles represents a novel, alternative mechanism by which soluble TNFR1 can be generated.

Role of Cyotkine Networks in Determining Susceptibility to Drug-induced Liver Disease (DiId). M. Holt[1], M. Bourdi[1], D. Eiras[1], T. Reilly[2], K. Welch[1], H. Amouzadeh[1], and L. Pohl[1]; [1]Molecular and Cellular Toxicology Section, [2]Bristol-Myers Squibb, Drug Safety Evaluation, Syracuse, NY.

Using interleukin (IL)-10 deficient mice (IL-$10^{-/-}$), we previously reported that IL-10 plays an important role in protecting the liver against acetaminophen (APAP)-induced liver injury (1). In the current work, we discovered a unique interaction that makes mice deficient in both IL-10 and IL-4 (IL-$10/4^{-/-}$) highly sensitive to the hepatotoxic effects of APAP. C57BL/6 wild type (WT), IL-$10^{-/-}$, IL-$4^{-/-}$, and IL-$10/4^{-/-}$ mice were administered APAP at a low dose of 120 mg/kg. Within 24 hours, 90 % of the IL-$10/4^{-/-}$ mice died of massive hepatic damage. In contrast, all of the WT, IL-$10^{-/-}$, and IL-$4^{-/-}$ mice survived and showed minor or no signs of liver injury. The high susceptibility of the IL-$10/4^{-/-}$ mice was associated with elevated levels of hepatic APAP-protein adducts, which appeared to be due in part to depressed levels of hepatic glutathione and to lower expression of γ-glutamylcysteine synthetase protein, but not elevated levels of CYP2E1. The high susceptibility of the IL-$10/4^{-/-}$ mice was also associated with elevated levels of hepatic TNF-a mRNA and serum nitrite/nitrate, which appeared to be due in part to depressed hepatic expression of arginase 1. These findings suggest that hepatocyte homeostasis following DILD is controlled in part by the activities of both IL-10 and IL-4, which together help maintain adequate levels of glutathione and prevent over expression of protoxicant factors including TNF-a and nitric oxide.

Membrin is a Golgi Membrane Receptor for Arf-1. A. Honda and J. G. Donaldson; Laboratory of Cell Biology.

ADP-ribosylation factor (Arf)-1 and Arf-6 are the least similar in amino acid sequence and the best-characterized members of the Arf family. Arf-1 is primarily localized to the Golgi complex where it regulates the assembly of cytosolic coat proteins and serves to regulate membrane traffic in the endoplasmic reticulum (ER)-Golgi system. Arf-6, by contrast, localizes to a novel, endosomal recycling system at the cell periphery. To identify sequences in Arf-1 that are necessary for Golgi localization, we examined the behavior of chimeric proteins between Arf-1 and Arf-6. As a result of this study, we identified the novel Golgi targeting motif (MXXE) in Arf-1 sequence, and we demonstrate that this sequence is recognized by membrin, an ER-Golgi SNARE protein. From these results, we propose that membrin is a Golgi membrane receptor for Arf-1.

Proteomic analysis of calcium-induced phosphorylation events in porcine heart mitochondria. R.K. Hopper, S. Carroll, and R.S. Balaban; Laboratory of Cardiac Energetics.

Post-translational modification of mitochondrial proteins by phosphorylation or dephosphorylation plays an essential role in Ca^{2+} signaling pathways involved in regulating energy metabolism and, potentially, in mitochondria-induced apoptosis. The purpose of this study was to screen for Ca^{2+} sensitive protein phosphorylations occurring in isolated mitochondria. Mitochondria isolated from porcine heart left ventricle were exposed to either no calcium or a high free calcium level (100 μM) to induce apoptosis, as confirmed by cytochrome c release. High [Ca^{2+}] was used in this initial screen for physiological as well as pathophysiological processes. After mitochondrial protein extraction the phosphoproteome was analyzed using 2-D gel electrophoresis and fluorescence staining using Pro-Q Diamond. More than 400 proteins were observed in the simple protein staining of the purified mitochondria preparation. Pro-Q stained phosphoproteins were partially sequenced and identified using MALDI tandem time-of-flight mass spectrometry. Numerous phosphorylated peptides (>50 individual proteins) were found under control conditions, including enzymes involved in mitochondrial energy metabolism including PDH, acyl-CoA dehydrogenases, and several site I, II, III and IV subunits. The degree of phosphorylation changed significantly in response to high levels of calcium for many proteins of varying function. Those currently identified include succinate dehydrogenase, citrate synthase, cytochrome oxidase, and manganese superoxide dismutase. The functional significance of the enzyme phosphorylations detected in this screen is currently being evaluated.

Amide Exchange Properties and Lifetimes of the Wild Type and Y54M vnd/NK-2 Homeodomain Bound to Two Consensus DNA Sequences. K.-J. Hwang, H.-S. Lee, J.-S. Maeng, J. M. Gruschus, J. W. Mack, J. H. Ju, K. Shahrokh, X.-P. Saunders, F. Bardon, M. Nirenberg, J. A. Ferretti ; Laboratory of Biophysical Chemistry.

The homeobox gene is important in specifying positional information and segmental identity in the commitment of embryonic cells to specific pathways of development. In the NK-2 class of homeodomains, our NMR studies have demonstrated that the conserved tyrosine in position 54 makes important contacts with the DNA, and these contacts have been shown to be critical to recognition of the unusual sequence of DNA that contains 5'-CAAGTG-3' as its core. The presence of methionine in position 54, which does not alter the structure of the homeodomain-DNA complex, does result in changes in the lifetime of the complex. In addition to these NMR structural and dynamics studies, we have performed CAT assays and transgenic experiments in *Drosophila melanogaster* to examine the functional effects of amino acid replacements in position 54 and to relate the corresponding results to the observed molecular level alterations. The hydrogen-deuterium exchange measurements, which reflect protein dynamics, represent one way to obtain important information on the stability of the homeodomain-DNA complex. Our deuterium exchange data on four different systems implies an important relationship between the lifetimes of the homeodomain-DNA complex and

their functionality, even with minimal structural changes. Residue W48 has the longest life time in the vnd/NK2-DNA complex, which indicates it forms the core of the most stable folded parts of the protein. The results show that the lifetime of the wild type analog is at least one order of magnitude longer than the corresponding mutants, all of which lead to embryonic lethality in *D. melanogaster*.

ATM is Activated by High NaCl, Contributing to Increased TonEBP/OREBP-mediated Transcription. C.E. Irarrazabal, M.B. Burg, and J.D. Ferraris; Laboratory Kidney Electrolyte Metabolism.

High NaCl activates the transcription factor, TonEBP/OREBP, increasing transcription of several genes, including BGT1. High NaCl also damages DNA, and DNA damage activates ATM kinase through autophosphorylation on Ser1981. TonEBP contains ATM consensus phosphorylation sites around serines 1197, 1247 and 1367. The present studies on HEK293 cells test whether ATM is involved in activation of TonEBP by high NaCl (osmolality raised from 300 to 500 mosm). Results: 1) Wortmannin, an ATM inhibitor, reduces high NaCl-induced activation of an ORE reporter and increase of BGT1 mRNA. 2) Overexpression of wild type (wt) TonEBP at 300 mosm increases the reporter activity, but TonEBP S1197A, S1247A or S1367A increases it much less. 3) High NaCl activates ATM, indicated by a large increase in S1981 phosphorylation (Western analysis). 4) Although ionizing and ultraviolet radiation damage DNA and activate ATM, they do not increase reporter activity. 5) TonEBP reciprocally co-immunoprecipitates with ATM kinase, demonstrating physical association. 6) AT cells (in which ATM is inactive) have reduced hypertonicity-dependent activation of reporter activity. The defect is complemented by wt, but not S1981A ATM. 7) Wortmannin further decreases high NaCl-induced increase in reporter activity in AT cells, consistent with participation of other signaling kinases. Interpretation: signaling via ATM is necessary for high NaCl-induced activation of TonEBP, but is not sufficient.

Identification of NEFA as an ARTS-1 Interacting Protein that Promotes the Release of Soluble TNFR1. A. Islam, S. J. Levine; Pulmonary Critical Care Medicine Branch.

Tumor necrosis factor is a pro-inflammatory cytokine that has been implicated in the pathogenesis of asthma, COPD, pulmonary fibrosis, and sarcoidosis. Excessive tumor necrosis factor (TNF) bioactivity is regulated by the generation of soluble TNF receptors that function as soluble TNF binding proteins. ARTS-1 (Aminopeptidase Regulator of Type I TNF Receptor Shedding), a type II integral membrane protein expressed by human airway epithelial and vascular endothelial cells, binds to and promotes the release of soluble TNFR1. We hypothesized that the mechanism by which ARTS-1 promotes the release of soluble TNFR1 involves ARTS-1-interacting proteins. Therefore, a yeast two-hybrid analysis was performed, utilizing the ARTS-1 extracellular domain as bait, which identified the calcium binding protein, NEFA (DNA-binding/EF-hand/Acidic amino acid-rich protein), as an ARTS-1-interacting protein. RNA interference, in the form of exogenously administered short interfering RNA sequences (siRNA), was utilized to investigate whether NEFA is required for the release of soluble TNFR1. A NEFA-specific siRNA

was developed which selectively down-regulated NEFA mRNA expression in human vascular endothelial cells (HUVEC) by 73%, as determined by quantitative, real time RT-PCR (mean 1.36±0.4 vs. control group mean 5.07±1.6, arbitrary units for NEFA mRNA expression; $p<0.05$). HUVEC cells treated with NEFA siRNA for 4 days demonstrated a 61% decrease in the quantity of TNFR1 released into culture supernatants as compared to cells treated with or without control siRNA (mean 40.3±0.4 vs. control group mean 103.1±4.1, pg/mL; $p<0.05$). In conclusion, we have identified NEFA as an ARTS-1-interacting protein that promotes the constitutive release of soluble TNFR1. We propose that the mechanism by which soluble TNFR1 is constitutively generated involves a direct protein-protein interaction between ARTS-1 and NEFA.

Tissue and Cellular Distribution of an Inserted Isoform of Nonmuscle Myosin II-C. S. S. Jana, S. Kawamoto, R. S. Adelstein; Laboratory of Molecular Cardiology.

Previous work from this laboratory has revealed the presence of an exon of 30 nucleotides encoding 10 amino acids that can be spliced into the heavy chain of nonmuscle myosin II-B (NMHC II-B) in loop I near the ATP binding domain. Although the noninserted isoform of NMHC II-B has a wide cellular distribution throughout the body, the inserted isoform is only expressed in neuronal cells. The newly recognized NMHC II-C also contains an alternative exon composed of 24 nucleotides encoding 8 amino acids. This exon, similar to that present in NMHC II-B, is spliced into the same locus in loop I, but unlike the neuronal-specific II-B isoform, is present in a wide variety of mouse tissues. These include liver, kidney and testis, where RT-PCR analysis reveals that the mRNA encoding the inserted form is greater than 90% of the total mRNA, as well as brain and lung, where it is about 50%. Adult mouse skeletal and cardiac muscle tissues are devoid of the inserted NMHC II-C isoform, but human skeletal muscle NMHC II-C mRNA is more than 50% inserted isoform. The expression of the inserted isoform changes during mouse development, being 50% at embryonic stage 14 and rising to approximately 90% after birth in the liver and kidney. Interestingly, we have examined one case of human colon carcinoma which was over 90% inserted mRNA, whereas mRNA from normal colon was practically all noninserted.

Tissue Absorbance and Heating in Two-Photon Microscopy of the Heart. P. Jobsis, E. Rothstein, C. Combs, and R. Balaban; Laboratory of Cardiac Energetics.

Near infrared (NIR) light has lower energy photons, less tissue absorbance and scatter than lower wavelengths. The low scattering and absorbance permit deeper penetration, and attenuate the non-specific photodynamic effects outside of the two-photon condition region. However, many *in vivo* tissues contain NIR absorbing chromophores, such as hemoglobin, myoglobin and cytochrome *c* oxidase, at much higher concentrations than cultured systems. These absorbances have generally not been considered in previous models of tissue heating in two-photon microscopy and its effects. To evaluate the contribution of tissue chromophores to NIR heating, absorbance of heart tissue was measured from 700-

900 nm (see figure) In porcine heart, tissue absorbance values of 0.408 ±0.14, 0.257 ±0.07 and 0.247 ±0.43 OD cm-1 were measured at 700, 800 and 900 nm, respectively. These absorbance levels represent 15,400%, 2,300% and 500% increases over that of water alone. At laser power of 100mW, this could result in a 3 to 20 ^{o}C rise in temperature at the focal spot alone depending on the wavelength and mathematical model used. These values suggest that direct temperature, or tissue viability, measurements under two-photon conditions, in vivo, are warranted.

Inhibition of Apoptosis in Acute Promyelocytic Leukemia Cells Leads to Increase in Levels of Oxidized Protein and LMP2 Immunoproteasome. M. A. S. Khan, H. Oubrahim, and E. R. Stadtman; Laboratory of Biochemistry.

Upon reaching maturity animal organs cease to increase in size, due to inhibition of cell replication activities. It follows that maintenance of optimal organ function is dependent upon the elimination of oxidatively damaged cells and their replacement with new cells. To examine the effects of oxidative stress and apoptosis on the accumulation of oxidized proteins we exposed acute promyelocytic leukemia cells to arsenic trioxide in the presence and absence of a general caspase inhibitor (z-VAD-fmk), which is known to inhibit caspase induced apoptosis. We confirm that treatment of cells with arsenic trioxide induces apoptosis and leads to the generation of oxidized proteins. Furthermore, inhibition of caspase activities prevented arsenic trioxide induced apoptosis, and led to a substantial increase in the generation of oxidized proteins. Moreover, inhibition of caspase activity in the absence of arsenic trioxide led to elevated levels of LMP2 and LMP7 immunoproteasome proteins. We show also that caspase inhibition leads to increases in the levels of oxidized proteins obtained by treatments with H2O2 + ferrous iron. Collectively, these results suggest the possibility that an age-related loss in capacity to carry out apoptosis might contribute to the observed accumulation of oxidized proteins during aging and age-related diseases.

Characterization of the Ezymatic Function of Nonmuscle Heavy Meromyosin II-B and II-C. K. Y. Kim, Y. A. Preston, M. Kovacs, E. V. Harvey, J. R. Sellers, and R. S. Adelstein; Laboratory of Molecular Cardiology.

We recently generated homozygous mutant mice harboring a single amino acid mutation (R709C) in the nonmuscle myosin (NM)-IIB allele. These mice showed a major decrease in the migration of certain neuronal cells and an increase in cardiac myocyte binucleation and cell size. Of interest, this site is conserved in smooth and nonmuscle myosins from vertebrates and is present in the head domain of myosin. Replacement of the homologous Arg with a Cys residue is known to occur in the human NM-IIA molecule (R702C) resulting in patients with May-Hegglin Anomaly and Fechtner syndrome and is associated with abrogation of NM-IIA function. In addition, mutation of the same amino acid in NM-IIC caused an autosomal dominant hearing loss in humans. NM-IIC contains an alternatively spliced exon of 24 nucleotides in loop I and the expression of inserted (IIC1) and noninserted (IIC) isoforms have different distributions in a variety of tissues. However, the functional consequences of the R709C in NM-IIB and the alternatively spliced isoforms in NM-IIC have not been studied. Therefore we characterized the

in vitro activity of NM-IIB and NM-IIC using the baculovirus system to coexpress heavy meromyosin (HMM) including the appropriate light chains. The in vitro activities of mutant IIB myosins (R709C-HMM) were compared with that of wild type myosins (WT-HMM). The MgATPase activity of phosphorylated WT-HMM was markedly activated by actin (0.17±0.05 s-1), whereas the activity of the R709C-HMM was activated to a lesser extent (0.05±0.007 s-1). The Vmax of R709C-HMM was about 29.4% of that of WT-HMM and its KATPase was significantly lower than that of WT-HMM. WT-HMM translocated actin filaments at a rate of 0.18 /s when phosphorylated. In contrast, using phosphorylated R709C-HMM, we could not observe any movement of actin filaments in the *in vitro* motility assay, but actin filaments did remain bound to HMM. Characterization of the actin activated MgATPase activity of phosphorylated IIC-HMM and IIC1-HMM demonstrated a V_{max} of 0.34±0.12 s^{-1} and 0.55±0.18 s^{-1}, and K_{ATPase} of 15.9±0.73 and 32.3±1.82 mM, respectively. Phosphorylated IIC-HMM translocated actin filaments at about one-half the velocity of IIC1-HMM (0.075±0.012 mm/s). Therefore, we suggest that the R709C mutation of NM-IIB results in defective activity of myosin II-B in the homozygous mutant mice, and that the inserted and noninserted isoforms of NM-IIC have different activities in a variety of tissues.

Arf6 regulates Rac1-induced ruffling by interacting with human kalirin in Hela. T.H. Koo and J.G. Donaldson; Laboratory of Cell Biology.

ADP-ribosylation factor (Arf)-6 regulates membrane trafficking and actin cytoskeleton at the plasma membrane by cycling GDP-GTP states and interacting with other proteins. To elucidate the functional role of Arf6, we have found several Arf6-interacting proteins by yeast 2-hybrid system. Of these, we determined human kalirin, a Dbl family member, interacted with Arf6 by GST pull down assay and competition/rescue assay. By constructing mutants, we confirmed that the spectrin-repeat domain of human kalirin was responsible for the interaction with Arf6 and Dbl homology (DH) – Pleckstrin homology (PH) domain was responsible for Rac1 activation in Hela cells. Next, we demonstrated that the expression of Arf6 T27N or depletion of Arf6 by RNAi treatment inhibited human kalirin-induced ruffling. Through these results, we suggest that Arf6 can regulate Rac1-induced ruffling at the plasma membrane by interacting with human kalirin.

Unique Mechanism of Action of Myosin-X, a Membrane Motor. M. Kovacs, F. Wang, J. R. Sellers; Laboratory of Molecular Cardiology.

Myosin-X is a membrane-attached motor playing an essential role in phagocytosis and axonal regeneration by propelling membrane protrusions. We investigated the interplay of myosin-X's interaction with actin, ATP and ADP using spectroscopic, steady-state and transient kinetic techniques and propose a unique kinetic model that is different from those of both myosin-II working in thick filaments and the vesicle transporters myosin-V and VI capable of moving along actin as single molecules. Spectral changes on binding of myosin-X to pyrene-labeled actin indicate a different mode of actin interaction from other myosins. Upon ATP-induced actomyosin dissociation, the kinetics of the pyrene fluorescence change is much

faster than the decrease in light scattering. This unique behavior indicates that a transition from a strongly to a weakly actin-bound state of myosin-X well precedes the actual detachment from actin. Thus, a weakly (non-stereospecifically) actin-attached state that may slide along actin will have a notably long lifetime. Rapid chemical quench shows that ATP hydrolysis occurs in this state, which is highly unusual compared to other myosins. Myosin-X exchanges ADP rapidly but has a very high ADP affinity that, under certain conditions, allows a much more effective product inhibition (i.e. stalling of the motor on actin) than in other myosins. A high time-averaged actin attachment is necessary for a low-density membrane motor to support continuous movement. Myosin-X achieves this by a unique mechanism whereby it remains attached to actin throughout most or all of its enzymatic cycle, while flipping between weak (sliding) and strong (fixed) actin-bound states.

The DNA Binding Agents Hoechst 33258 and 33342 Enhance Recombinant Adeno-Associated Virus (rAAV) Transgene Expression. L. Li, L. Yang, R. M. Kotin; Laboratory of Biochemistry Genetics.

Recombinant adeno-associated viruses (rAAV) are commonly used in pre-clinical and clinical gene transfer studies. However, the relatively slow kinetics of rAAV transgene expression complicates *in vitro* and *in vivo* experiments. Our results revealed that two different, although related, DNA Minor Groove binding drugs Hoechst 33258 and 33342 accelerate the kinetics of rAAV transgene expression. We found that Hoechst 33258 and 33342 increase both the level of reporter gene expression and the population of gene expressing cells by transduction of rAAV2-EGFP in 293 cells. In addition, the augmentation of rAAV2 gene expression occurs in different cell types in a concentration dependent manner. We also found that gene expression of different serotypes of rAAVs, such as rAAV-1 and rAAV-5, enhanced by Hoechst 33258 and 33342. The Hoechst 33258 or 33342 mediated enhancement of rAAV gene expression correlated with an increase of cells in S phase and G2/M phases of the cell cycle. To study the mechanism, in vitro transcription assay was then used. We found that Hoechst 33258 or 33342 enhanced rAAV gene expression by increasing transcription while other DNA minor groove binders have no such effect. These findings suggest that the accelerated the kinetics of rAAV transgene expression by Hoechst 33258 and 33342 might extend the applications of rAAV for gene transfer and Hoechst agents might be useful for gene therapy applications since they are potential enhancer drugs for gene expression.

Sumoylation of Heterogeneous Nuclear Ribonucleoproteins, Zinc Finger Proteins, and Novel Nuclear Pore Complex Proteins. T. Li, P. B. Chock; Laboratory of Biochemistry.

SUMO, a small ubiquitin-related modifier, is known to covalently attach to a number of nuclear regulatory proteins such as p53, PML, c-Jun and c-Fos. The sumoylation reaction is catalyzed by the SUMO protease which exposes the C-terminal active glycine residue of the nascent SUMO, the heterodimeric SUMO activating enzyme, the SUMO conjugating enzyme, Ubc9, and SUMO protein ligases, in a manner similar to ubiquitinylation. Identification of SUMO regulated proteins is hampered by the fact that many sumoylated proteins are present at a

level below normal detection limit. This limitation was overcome by either *in vivo* overexpression of Myc-SUMO or i*n vitro* sumoylation with excess biotin-SUMO and Ubc9. Sumoylated proteins so obtained were affinity purified or isolated by immunoprecipitation. The isolated sumoylated proteins were identified by sequence analysis using mass spectrometric methods. Results reveal that several heterogeneous nuclear ribonucleoproteins (hnRNPs), zinc finger proteins and nuclear pore complex proteins were sumoylated. The sumoylation of hnRNP A1, hnRNP F and hnRNP K were confirmed *in vivo* by co-immunoprecipitation. In views of the facts that hnRNPs have been implicated in RNA splicing, transport, stability, and translation, our findings suggest that sumoylation could play an important role in regulating mRNA metabolism.

Identification of Human Methyl-CpG Binding Domain Protein (MBD) 4 as a Substrate of Protein Kinase X. W. Li, R. M. Kotin; Lab of Biochemical Genetics.

The human protein kinase X (PrKX) is an X chromosome encoded protein belonging to the family of cAMP-dependent protein kinases. Although PrKX shares some characteristics with PKA, several studies have demonstrated functional differences between PrKX and PKA. Based on these differences, we postulated that the substrates of PrKX and PKA may differ. To identify the substrates of PrKX, we employed a yeast two-hybrid screen using PrKX as the bait. The human protein MBD4 was isolated as a strong interaction partner. The stability and specificity of interaction were confirmed by *in vitro* pull-down experiments and immunoprecipitation of complexes assembled *in vivo*. Subsequently, we showed that the hMBD4 protein expressed in E. coli could be phsosphrylated by PrKX as well as PKA, CKII and PKC. Phosphorylation of hMBD4 by PrKX, PKA, CKII and PKC modulated the binding activity of hMBD4 to fully methylated DNA.

Identification of Promoter Elements in Mouse PDE3B Gene: The Role of CREB in PDE3B Expression. H. Liu, J. Tang, V. C. Manganiello; Pulmonary Critical Care Medicine Branch.

To understand mechanisms for transcriptional regulation of murine cyclic nucleotide phosphodiesterase 3B (MPDE3B) expression, we incubated 3T3-L1 preadipocytes with isobutylmethylxanthine (IBMX), dexamethasone and insulin, alone or in combination, and found that IBMX, which increased CREB phosphorylation, was the predominant regulator of MPDE3B expression. Real-time PCR and immunoblotting analysis indicated that ectopic expression of dominant-negative adeno-KCREB in 3T3-L1 cells markedly inhibited MPDE3B mRNA and protein inductions by IBMX. Using various luciferase constructs containing different fragments of the 5'-flanking region of MPDE3B gene, we identified a distal promoter that contained CRE cis-acting elements and induced about 50-fold higher luciferase activity than did a proximal, GC-rich and TATA-less promoter. Mutation of the CRE sequences dramatically reduced distal promoter activity. Electrophoretic mobility shift analysis indicated that CRE-binding was greater in nuclear extracts from differentiating adipocytes than from preadipocytes. Chromatin immunoprecipitation (ChIP) analyses with antibodies specific to CREB, phospho-CREB, and ATF-1

showed that these CREB family members associated with the distal promoter, and that interaction of phospho-CREB, the active form of CREB, with the MPDE3B gene promoter was increased in IBMX-treated 3T3-L1 cells. Taken together, our results indicate that CRE elements in the distal promoter region and activation of CREB proteins might play a crucial regulatory role in MPDE3B expression during preadipocyte differentiation. Transcription initiation site mapping suggested that the distal promoter might function as an enhancer whereas the proximal promoter drives transcription of the MPDE3B gene.

Activation of Synoviocytes by the Phospholipase Motif of Parvovirus B19 Capsid Protein (VP1): J. Lu, N. Zhi, and K. E. Brown; Hematology Branch.

Parvovirus B19 (B19) infection in adults is often associated with an acute symmetrical polyarthropathy but the mechanism is unclear. Using IF and RT-PCR we confirmed that B19 does not infect human fibroblast-like synoviocytes (HFLSs). However, using a modified Boyden chamber migration assay, B19 induced HFLS migration in vitro, suggesting a direct role of the viral capsid in synoviocyte activation. B19 has two capsid proteins: the minor capsid protein VP1, which comprises 5% of the virus, is identical to VP2 with an additional 227aa at the carboxyl terminus, the VP1 unique region (VP1u). Recent studies have shown that B19-VP1u has a conserved phospholipase motif. Expression of B19-VP1u in E. coli confirmed that the protein had secretory phopsholipase activity and induced synoviocyte migration. Proteins with point mutations in the critical amino acid of phospholipase motif were non-functional in both assays. Prostaglandins (PG) are associated with arthropathy and produced from arachidonic acid by phospholipases. We investigated the effects of VP1u on PGE2 release in cultured HFLSs by EIA, and showed a dose-dependent increase in PGE2 levels in supernatant of cells cultured with VP1u but not with the mutants. A dose- and time-dependant increase in COX-2 but not COX-1 (constitutive gene) expression was demonstrated by WB and real-time RT_PCR. Our data indicate that the HFLSs can be activated by the phospholipase motif of B19 VP1, and the activation of the synoviocyte by B19 is infection-independent. These data support this as part of the mechanism by which B19 induces acute polyarthropathy in patients, and could provide a basis for the development of new therapeutic tools to control B19-induced arthropathy.

Quick Quantification of Coomassie-Stained Proteins in SDS-PAGE Gels at Nanogram Sensitivity Using Infrared Fluorescent Assay. S. Luo and R. L. Levine; Laboratory of Biochemistry.

A new and quick method for quantification of Coomassie-stained proteins in SDS-PAGE gels is developed which utilizes the infrared fluorescence of Coomassie blue. The Coomassie-stained gels were visualized with the Odyssey Infrared Imaging System developed at LI-COR. The imager uses lasers to generate excitation light at 680-nm, and detects emitted light at 720-nm. We discovered that this system is not only able to image the gels, but also to quantitate the stained proteins. Moreover, because the classical staining-destaining procedures, which usually take overnight, are designed for visible detection methods, but Odyssey imager shows very high sensitivity, so we developed an improved procedure for

staining and destaining, which gives good results for quantification by using Odyssey imager. The new procedure uses a total of 30-min post-electrophoretic treatment, which including 10-min staining with lower concentration of Coomassie Blue R-250, 10-min destaining, and 10-min washing with water, and then the gels are ready for quantification. By using this improved procedure and Odyssey imager, it is able to detect as high as 20-microgram of target protein in the SDS-PAGE gels, and also to detect as low as 10-ng of protein - a sensitivity level that matches western blot. Coomassie Blue G-250 stained proteins in the SDS-PAGE gels are also good for quantification by Odyssey system. For application, we were able to determine the specific activity of a recombinant protein in the cell extract before purification by quantitating the protein separated by SDS-PAGE and doing the activity assay on the same cell extract.

Inducible Mitochondrial Uncoupling – An Endogenous Regulatory Mechanism Augmenting Tolerance to Cardiac Ischemia. C. J. McLeod[1], J. P. McCoy Jr[2], R. F. Hoyt Jr[3], M. N. Sack[1] ; [1]Cardiovascular Branch, [2]Flow Cytometry Core, [3]Laboratory of Animal Medicine and Surgery.

Angina pectoris or experimentally controlled transient cardiac ischemic one day prior to a myocardial infarction improves clinical outcome and attenuates infarct-size respectively. This biological phenomenon is termed delayed ischemic preconditioning and constitutes the transactivation of a cell-survival program in response to 'trigger' ischemia-activated signaling. The functional genomic control underpinning the ischemia-tolerant phenotype of delayed preconditioning remains elusive. We demonstrate that cardiac enriched uncoupling proteins 2 and 3 are upregulated in response to delayed ischemic preconditioning. In parallel, preconditioned mitochondria exhibit modest inducible uncoupling of oxidative phosphorylation with concomitant partial inner mitochondrial membrane depolarization. Moreover, preconditioned mitochondrial suppress anoxia-reoxygenation mediated mitochondrial reactive oxygen species generation and display enhanced anoxia-reoxygenation tolerance compared to non-preconditioned mitochondria. Collectively, these data implicate that uncoupling proteins are endogenously up-regulated in the heart as an adaptive program to attenuate ischemia-reperfusion injury. Modulation of uncoupling proteins may be a novel approach to preemptively protect various tissues against ischemic injury.

Nuclear Localization and Molecular Partners of Big1, a Brefeldin A-inhibited Guanine Nucleotide-exchange Protein for Adp-ribosylation Factors. P. I. Padilla, G. Pacheco-Rodriguez, J. Moss, and M. Vaughan; Pulmonary Critical Care Medicine Branch.

BIG1 is a ca. 200-kDa, brefeldin A-inhibited guanine nucleotide-exchange protein that preferentially activates ARF1 and ARF3. It was initially purified from bovine brain cytosol in a multimolecular complex with a similar ARF-activating protein, BIG2, which is also an A kinase-anchoring protein. In HepG2 cells growing with serum, BIG1 was primarily cytosolic and Golgi-associated. After incubation overnight without serum, a large fraction of endogenous BIG1 was in the nuclei. By confocal immunofluorescence microscopy, BIG1 was localized with nucleoporin p62

at the nuclear envelope (probably during nucleo-cytoplasmic transport) and in nucleoli, clearly visible against the less concentrated overall matrix staining. BIG1 was also identified by Western blot analyses in purified subnuclear fractions (e.g., nucleoli and nuclear matrix). Antibodies against BIG1, nucleoporin, or nucleolin co-immunoprecipitated the other two proteins from purified nuclei. Unlike their localization in cytoplasm, BIG1 and BIG2 were not associated in nuclei. Also of note, ARF was never detected among proteins precipitated from purified nuclei by anti-BIG1 antibodies, although microscopically the two proteins did appear sometimes to be co-localized in the nucleus. These data are consistent with independent intracellular movements and actions of BIG1 and BIG2. They are also evidence of the participation of BIG1 in both Golgi and nuclear functions.

A Comparison of Time-Resolved Anisotropies of Two Fluorescent Guanosine Analogs in Single and Double Stranded DNA. K. W. Poulin, M. E. Hawkins*, F. M. Balis* and J. R. Knutson; Laboratory of Biophysical Chemistry, *Pediatric Oncology Branch, NCI. Pteridine guanosine analogs are highly effective fluorescent probes for monitoring DNA structures and DNA-protein interactions. These analogs have a relatively high quantum yield and like the well-characterized fluorescent probe 2-aminopurine (2-AP), the analogs base stack within the DNA making them native-like with neighboring bases. The pteridine guanosine analogs, 3-methylisoxanthopterin (3-MI) and 6-methylisoxanthopterin (6-MI) were directly incorporated into the sequence: 5‘-act aga gat ccc tca gac cct ttt agt cag tFt gga aaa tct cta gca gt-3’ where F denotes the position of the 3-MI or 6-MI. In comparisons of melting temperatures of 3-MI or 6-MI-containing sequences, it is apparent that 6-MI forms much more stable duplexes. This is most likely due to the steric hindrance from a methyl group in the 3-position of 3-MI precluding hydrogen bonding. We have measured fluorescence intensity, time resolved anisotropy, steady state lifetimes, and decay-associated spectra of these single and double stranded sequences. The data confirm that 6-MI more clearly reports the global motions of the single or double strands. In contrast, 3-MI exhibits local motions which may represent localized unwinding and/or ‘base flipping’.

Suppression of Toll-Like Receptor (TLR) Mediated Activation of Mast Cells by Dexamethasone. H. Qiao and M. A. Beaven; Laboratory of Molecular Immunology

Mast cells play a critical role in innate immune response to bacterial infection by production of inflammatory cytokines1,2 probably through the interaction of bacterial cell wall components and TLRs3,4. An question related to treatment of allergies is whether glucocorticiods suppress responses to TLR activation. Here we have investigated the effects of the glucocorticoid, dexamethasone,on intracellular signaling pathways and responses activated by the synthetic TLR2 ligand, tripalmitoyl Cys-Ser-(Lys)4 (Pam3Cys), in the MC/9 mast cell line. These cells were found to express TLRs as well as associated signaling molecules and respond to Pam3Cys via TLR2 to induce the generation of inflammatory cytokines but not degranulation or release of arachidonic acid. Other responses included activation of IRAK1, JNK, p38 MAP kinase and downstream transcription factors such as NFκ B and c-Jun. These activations as well as production of TNFa, IL-6, and IL-13 were

suppressed by prior incubation of cells with low concentrations of dexamethasone (50% inhibition at $\leq$10nM). A notable exception was that activation of IRAK1 was not suppressed by dexamethasone to indicate that dexamethasone acted downstream of IRAK1. Comparision with antigen stimulation indicated that antigen-mediated production of TNFα and related signaling events were also suppressed by the same low concentrations of dexamethasone. These data suggested that dexamethasone suppresses responses to both antigen and Pam3Cys even though the initial signaling pathways for these two stimulants are different. In view of the perceived role of TLRs in innate immune responses the use of glucocorticoids in the treatment of allergies may have detrimental side-effects by minimizing inflammatory protective reactions to bacterial or parasitic infection.

Reduction of TFII H Complex is Associated with Global Down-regulation of Alveolar Macrophage Transcription and Gene Expression in Idiopathic Pulmonary Fibrosis. P. Ren, I. Rosas, H.P. Wu, S.D. MacDonald, B.R. Gochuico; Pulmonary Critical Care Medicine Branch.

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease of unknown etiology. We previously reported that global down-regulation of gene expression in alveolar macrophages was associated with low concentrations of TFII H p89 and p62 subunits. We hypothesized that low concentration of TFII H p89 and p62 subunits would affect the formation of the TFII H complex and lead to decreased basal transcription activity in alveolar macrophages of IPF patients. Total cell and nuclear extracts of alveolar macrophages were purified from broncho-alveolar lavage fluid from IPF patients and normal volunteers. Co-immuno-precipitation and 2-D gel electrophoresis of total cell extracts were used to determine formation of the TFII H complex. Basal transcription activity of nuclear extracts from alveolar macrophages was quantified using an in vitro transcription system. Immuno-precipitate using anti-TFII H p44 antibody contained TFII H p44, p89 and p62. Precipitated TFII H p89 and p62 were significantly lower in alveolar macrophages from IPF patients compared with normal volunteers ($p=0.023$ and $p=0.042$, respectively). Consistent with these data, functional analysis demonstrated that basal transcription activity was reduced approximately 41% in IPF patients. Our findings demonstrated that reduction of TFII H complex is associated with low basal transcription, which may contribute to global down-regulation of gene expression in alveolar macrophages of IPF patients.

Two-Photon Imaging of Muscle in the Living Mouse. E. Rothstein, P. Jobsis, C. Combs, and R. Balaban, Laboratory of Cardiac Energetics.

In vivo, two-photon microscopy provides the opportunity to investigate intercellular events within the living animal. These studies were conducted to evaluate probes for following regional mitochondrial energetics and plasma membrane potentials in the intact animal using intrinsic NADH and di-8-ANEPPS ("fast" potentiometric probe) as two-photon imaging probes in the living mouse. Anesthetized mice (2.5% isofluorane) were injected with 1 mM di-8-ANEPPS in DMSO with 0.05% Pluronic F-127 (Molecular Probes) administered via tail-vein at 7.5 ml/min/g over 2 minutes. The tibialis muscle was exposed for imaging. Using

a BioRad Radiance-2100, NADH or di-8-ANEPPS fluorescence was monitored (up to ~80microns deep). Di-8-ANEPPS did not interfere with the NADH measurements permitting both probes to be detected in the same animal. The NADH images revealed sub-cellular structures consistent with subsarcolemmal, interfibrillar and paranuclear mitochondria. Heterogeneity of the NADH signal in different cells suggested a variation in signal intensity in different muscle fiber types. In cells with intense NADH fluorescence signals, the structures of individual sarcomeres could be detected. Vascular voids were also observed. Di-8-ANEPPS staining was observed in the capillary system, outlining the muscle capillary structure and labeling the vessel wall. *Ex vivo* studies confirmed endothelial cell and sarcolemmal membrane labeling with di-8-ANEPPS. These data demonstrate the feasibility of using two-photon NADH and di-8-ANEPPS fluorescence in the skeletal muscle to monitor sub-cellular metabolic events as well as vascular structure with in the living mouse.

Myosin V Moves by a Hand-over-Hand Lever Arm Mechanism. T. Sakamoto[1], A. Yildiz[2], P. R. Selvin[2], J. R. Sellers[1]; [1]Laboratory of Molecular Cardiology, [2] Physics Dept. & Biophysics Center, University of Illinois, Urbana, IL, USA. Myosin V is a two-headed myosin with a long neck that has six IQ motifs complexed with calmodulin. It moved processively along an actin filament. Previously, we expressed HMM mutants in which the neck was either lengthened (8IQ) or shortened (4IQ) by altering the number of IQ motifs. The working stroke of single interactions was measured using optical trapping and the speed and processivity was determined by observing the movement of single fluorescently-labeled molecules on actin filament using total internal reflection fluorescent (TIRF) microscopy (Sakamoto et al. JBC 278: 29201, 2003). The results show that the working stroke and the speed at low KCl (25mM) of individual myosin V IQ mutants are proportional to neck length of myosin V. These results did not address the step size of the molecules taken during processive runs. To address this question, we are measuring the step size of all IQ mutants using FIONA (Fluorescent Imaging at One Nanometer Accuracy), which can determine the position of an individual myosin V fluorescently labeled at a single calmodulin residue with nanometer precision using TIRF microscopy (Yildiz et al. Science 300: 2061, 2003). Preliminary data show that the 4IQ mutant has a shorter step size and the 8IQ has a longer step size that obtained with wild type myosin V. These results strongly support a hand-over-hand lever arm model in which the step size is predominantly determined by the length of the IQ domain.

Detection, Isolation and Expression of ORFs of Novel TTV Genotypes. V.R. Shende and K.E. Brown; Hematology Branch.

TT virus, first identified in 1997 in a patient with seronegative hepatitis, is a member of a novel group of small non-enveloped, circular, ssDNA viruses. Subsequent studies showed that it comprises a heterogeneous cluster of viruses: over 28 genotypes have been described. To date, no association with human disease has been shown, although increased viral loads are seen in immunocompromised patients and children with bronchopneumonia. We identified

a healthy individual positive for TTV by PCR using conserved primers in the untranslated region (UTR). To obtain more sequence, DNA from plasma and bone marrow extracellular fluid was amplified by non-specific isothermic amplification (GenomiPhi, Amersham) and TTV specific PCR. Plasma DNA yielded a 2.9 kb fragment the bone marrow provided a 3.1 kb, both containing entire coding region. Sequence analysis revealed marked differences. The fragment from plasma has closest homology (50-60%) to genotype 11, 12 and 13 of group 3 strains while that from bone marrow was found close to strains in group 5 (Arch. Virol., 2002 147: 21-41). The N terminal 218aa of the ORF1 was expressed as V5/His tagged protein in Cos-7 and 293T cells using pcDNA3.1 vector (Invitrogen, CA). Our preliminary observation revealed nuclear localization of this protein right at 24 hr post transfection. Similar studies are planned to learn more about the way TTV ORFs function in infected cells. TTV DNA can be isolated from different body fluids however the titers are higher in the PBMCs and bone marrow. With real time quantitative PCR we tested serial blood draws from the donor. The plasma component of these was found to carry negligible, nearly 500 times less copies of TTV compared to the cells from the equivalent portion of blood indicating that the PBMCs might support replication of this novel virus. Further studies are on to find the exact cell type that supports its replication and to establish cell culture for TTV.

Requirement for Cx43a1 in heart development arises from an essential role in coronary vasculogenesis. D.L. Walker, M.L. Kirby, C.W. Lo; Laboratory of Developmental Biology.

Gap junctions, comprised of membrane channels called connexins, are conduits of cell-cell communication that mediate the intercellular passage of ions and small molecules. Connexin43 (Cx43a1) is the most abundant connexin in the mammalian heart and is required for normal heart development. The Cx43a1 knockout mouse dies at birth from outflow obstruction associated with infundibular pouches and coronary anomalies. Analysis using oligonucleotide microarrays indicated that 1275 genes are differentially expressed in the infundibular pouch of the Cx43a1 knockout (p-value < 0.001). Surprisingly, many transcripts identified by the microarray analysis are expressed by cells in the vasculature or are associated with vasculogenesis, including many vascular smooth muscle and endothelial cell markers, and genes in the TGFb and VEGF/Notch/Eph cell signaling pathways. Immunohistochemistry with antibodies to smooth muscle actin, von Willebrand Factor, PECAM, and collagen VIII together with studies carried out using an Sm22lacZ reporter confirmed the ectopic placement of vascular smooth muscle and endothelial cells in the pouch. Immunohistochemistry also showed elevated VEGF expression associated with the epicardium and endocardial cells lining the pouch. Interestingly, the pouch epicardium exhibited an unusual beaded rather than squamous epithelial cell morphology. A reduction in branching complexity was observed for the coronary arteries, with coronary arteries and veins consistently deploying towards the pouch malformations. Together, these findings indicate that abnormal vasculogenesis and remodeling of coronary arteries play a central role in the elaboration of the infundibular pouch phenotype in the Cx43a1 knockout mouse,

and suggest a previously unknown requirement for Cx43a1 gap junctions in coronary vasculogenesis.

Sulfation of Astrocyte-derived Proteoglycans is Essential for the Control of Neuronal Growth. H. Wang, Y. Katagiri, F. Tan, H. M. Geller; Developmental Neurobiology Group. The failure of injured axons to regenerate within the central nervous system after injury has been attributed to the formation of the glial scar. It is thought that molecules within the glial scar inhibit axon growth. Among these molecules, chondroitin sulfate PGs (CSPGs) are thought to have a primary role. Enzymatic removal of CSPG glycosaminoglycan (GAG) chains and blocking transforming growth factor-beta1 (TGF-beta1) each promote functional recovery after injury. Here we present data using cell culture models that 1) TGF-beta1-treated astrocytes are a model of the glial scar and 2) astrocyte-derived PG GAG chains and sulfation are essential for the inhibitory activity of CSPGs. Cerebellar neuronal growth on TGF-beta1-treated monolayers was reduced as compared to growth on untreated controls. TGF-beta1 upregulated the synthesis of CSPGs by astrocytes. Addition of pharmacological inhibitors of GAG chain synthesis or CSPG sulfation reduced this inhibitory activity, demonstrating that sulfation of CSPG GAG chains is essential for growth inhibition. The major CSPGs in the glial scar are 4- and 6- sulfated PGs. Quantitative RT-PCR indicated that TGF-beta1 upregulated RNA for chondroitin-4- sulfotransferase (C4ST) which produces 4-sulfated PGs, but not for C6ST. Western blot data using antibodies we made against both 4- and 6- ST confirmed that TGF-beta1 treatment induced C4ST, but not C6ST. We then prepared a purified spot of CS-A (a predominantly 4-sulfated sugar) on a substrate of poly-L-lysine (PLL), and plated neurons onto this substrate. We found that axons of neurons growing on PLL did not cross onto CS-A, but 4-sulfatase treatment of CS-A increased the percentage of neurons that crossed onto CS-A. FACE analysis of sugar structure analysis confirmed that 4-sulfate is removed by these sulfatases. We generated DsRed fusion proteins, with either WT or inactive forms of C4ST. Both fusion proteins were detected in the Golgi apparatus of transfected astrocytes. Neurons were plated onto these astrocytes. Overexpression of WT, but not inactive, C4ST, reduced the length of axons plated onto these astrocytes. In summary, these observations demonstrate that 1) TGF-beta1 induces the upregulation of CSPGs and 4-ST mRNA and protein, and 2) the sulfation composition and pattern of the GAG chains are critical to the inhibitory properties of astrocyte-derived CSPGs. 4-sulfated PGs are likely responsible for the inhibition of neuronal growth by TGF-beta1-treated astrocytes. These results also provide an assay system to screen for potential inhibitors of 4-sulfation which could be used in the therapy of spinal cord injuries.

Relative Quantification of Highly Complex Proteomes Using Liquid Chromatography and Nano-Spray Tandem Mass Spectrometry. G. Wang, W. W. Wu, W. Zeng, C.-L. Chou, and R.-F. Shen; Proteomics Core Facility, Hematology Branch, Laboratory of Kidney and Electrolyte Metabolism.

Stable isotope labeling is widely used in quantitative proteomics using LC-MS. By analyzing standard protein mixtures, we and others showed that comparison of chromatogram peak areas of peptides between different LC-MS runs could potentially be an alternative for relative quantification without isotopic labeling. In the present study, the reproducibility and linearity of such an approach were evaluated with highly complex proteomes, and its applicability to biomarker discovery demonstrated by identifying up- and down-regulated proteins in control and treated cells. A computer program was developed to automate the quantification process and provide statistical analyses. Various amounts of proteins from rat kidney or human plasma were subject to repeated LC-MS analyses. Highly reproducible data were obtained as evidenced by the near perfect Pearson correlation coefficients for peptide ion peak area and retention time between replicated runs. In many cases more than 50% of the measured peptide ions had an adjusted quantification error of less than 10%, and about 90% had an error of less than 20%. Within the dynamic range of the instrument, ratios of the added quantities of proteins correlated linearly with the calculated ratios of their chromatogram peak areas. To quantify the effects of EGF treatment on human A431 cells, six LC-MS runs for control and EGF-treated samples, respectively, were carried out. More than 100 proteins were identified in both samples, and among them about 10 were shown to have changed significantly. Similar results were obtained when the experiment was repeated. Overall, this study presented a highly reproducible approach to quantifying complex proteomes that can potentially be utilized in biomarker discovery.

Mechanistic Insights Revealed Through Characterization of a Novel Chromophore in Selenophosphate Synthetase from E. coli. M. D. Wolfe, T. C. Stadtman; Laboratory of Biochemistry.

The incorporation of selenium into specific proteins and tRNAs requires selenophosphate ($SePO_3$), whose formation is catalyzed by selenophosphate synthetase. In a Mg/ATP-dependent reaction, selenophosphate synthetase catalyzes the phosphorylation of selenide to yield AMP, inorganic phosphate, and $SePO_3$. In this report, a previously unrecognized chromophore covalently attached to selenophosphate synthetase is characterized. The UV/Vis spectrum of selenophosphate synthetase has a feature centered at 315 nm that is irreversibly destroyed by alkylation. Moreover, addition of Zn^{2+}, which is known to inhibit selenophosphate synthetase, reversibly quenches the 315 nm absorption. Since Zn^{2+} is known to bind to Cys17, these data strongly suggest that this residue participates in the 315 nm absorption. Upon incubation with *both* Mg^{2+} and ATP, the $lambda_{max}$ of the chromophore shifts to 340 nm, and it is shown that the shift requires binding of nucleotide having a hydrolyzable gamma-phosphoryl group. These data indicate that either the chromophore is directly involved in phosphoryl transfer or indirectly reflects a phosphorylation-dependent conformational change in selenophosphate synthetase. This work provides the first spectroscopic handle

on catalytic steps associated with $SePO_3$ synthesis, which will be used to study the molecular structure of the chromophore and its role in the catalytic mechanism of selenophosphate synthetase.

High Throughput Discovery of Mutations in Mouse Connexin Using Resequencing DNA Chip and Wave. Y. Wu, E. Doe, C. Rosenow*, C. W. Lo; Laboratory of Developmental Biology.

Connexins form gap junction channels that mediate the movement of ions and metabolites between cells. Although connexins have been shown to play an important role in heart development, the mechanism by which connexins modulate events in development is largely unknown. Particularly intriguing is the notion that connexins may have a signaling function that is separable from its membrane channel activity. To obtain further insights into connexin protein structure-function relationships and elucidate the role of connexins in cardiovascular development, we conducted a screen for connexin mutations in chemically mutagenized mouse embryonic stem cells. For this screen, we used two different high-throughput methods for mutation detection: a high density Affymetrix custom resequencing DNA microarray for obtaining sequence data on the coding sequences of all 20 mouse connexin genes, and also heteroduplex analysis using dHPLC analysis via the Transgenomic WAVE to detect mutation in four connexin genes expressed in the heart (Cx43, Cx37, Cx45, and Cx40 via). We have screened 960 ethyl methysulfonate (EMS) mutagenized embryonic stem cells and recovered five clones with connexin mutations - two in Cx43, one in Cx37, and two in connexin 45. Interestingly, the Cx37 mutation and two Cx45 mutations could not be identified by Sanger didoexy sequencing, although one of the Cx45 mutation was successfully identified using the connexin resequencing chip. Two of the ES cell clones were blastocyst injected to generate mice, and phenotype analysis of one of these mouse lines harboring a mutation in the intracellular loop of Cx43 revealed cardiovascular defects involving the outflow tract and right ventricular myocardium. This suggests that this region of the protein, though not involved in channel formation, has an important role in cardiovascular development. These studies suggest that banking a library of mutant connexin clones will be invaluable for defining connexin protein domains that play an essential role in cardiovascular development and disease.

Intracellular Localization of Myosin18A. Y. Yang, Q. Xu, and J. Sellers; Cell Motility Group.

Mouse myosin18A, containing a KE and a PDZ domain at the N-terminal is an unconventional myosin which was first cloned from bone marrow stromal cells. The protein expression level was positively related with the supportive ability of stromal cells. To date, there are at least four myosin18A isoforms in mouse brain, one of which is missing the KE and PDZ domains. We prepared an antibody against a peptide from C-terminus of the myosin. Using this antibody myosin18A was immunoprecipitated from adult mouse brain. The antibody specificity was determined by LC-Mass spectrography of the immunoprecipitated high molecular weight band. Immuno-histochemical staining suggests that myosin18A is also expressed in adult mouse brain. On whole mount embryo section the staining is

seen predominantly in epithelial cells. To understand the detailed localization of myosin18A in epithelial cell, we produced a GFP-tagged myosin18A heavy chain construct. After transfection, we find that Myo18A-GFP localizes at the lamellipodia in mouse spleen epithelial cells and co-localizes with F-actin at lamellipodia. Some myo18a-GFP forms into dot structures in the cell body. These results indicate that myosin18A might bind with other molecules or organelles and organize into macro-molecules in cells.
To study the kinetics of myosin18A, we also created a couple of constructs: myosin18A HC, myosin18A HC without KE-PDZ domain and HMMs to be expressed in Sf9 cells.

Exchange of Clathrin on Clathrin-Coated Pits in Permeabilized Tissue Culture Cells. Y.I. Yim, S. Scarselletta, F. Zang, D.-W. Lee, L. Greene and E. Eisenberg; Laboratory of Cell Biology.

Clathrin and clathrin adaptors on clathrin-coated pits exchange with cytosolic clathrin and clathrin adaptors *in vivo* and this exchange might require the molecular chaperone Hsc70 and J-domain protein auxilin, which, with ATP, uncoat clathrin-coated vesicles both *in vivo* and *in vitro*. We now show, using permeabilized cells, that a mixture of Hsc70, auxilin, and ATP causes rapid dissociation of clathrin but not clathrin adaptors from clathrin-coated pits, and, even when free clathrin is also present, clathrin exchange does not occur. In contrast, cytosol dissociates both clathrin and clathrin adaptors from pits *in vitro*, and concomitant with this dissociation, clathrin and clathrin adaptors rebind to the same pits establishing that, as *in vivo*, these proteins exchange *in vitro*. Since both dissociation and exchange of clathrin *in vitro* are prevented by inhibiting Hsc70 activity, we conclude that Hsc70 and auxilin are required for the clathrin-exchange that occurs on clathrin-coated pits *in vivo*.

Efficient Recovery of Mouse Mutations Causing Congenital Cardiovascular Anomalies. Q. Yu, Y. Shen, B. Chatterjee, B. Siegfried, L. Leatherbury, C. W. Lo; Laboratory of Developmental Biology.

To elucidate the genetic basis for congenital heart disease, we conducted a noninvasive fetal ultrasound screen of N-ethyl N-nitrosurea (ENU) mutagenized mice to identify mutants with cardiovascular anomalies. Ultrasound interrogation of 7546 fetuses from 262 families yielded 399 fetuses from 124 families with cardiovascular defects. Assuming a mutation load of 20-50 per family, these results would provide a minimal estimate of 1-2% of the mouse genome being essential for cardiovascular development. Further analysis showed a variety of congenital cardiovascular anomalies, including persistent truncus arteriosus (PTA), interrupt aortic arch (IAA), transposition of the great arteries (TGA), double outlet right ventricle, Tetralogy of Fallot, pulmonary atresia, ventricular/atrioventricular septal defect, coronary anomalies and hypertrophic cardiomyopathy. Most defects are heritable as recessive mutations. Using polymorphic microsatellite DNA markers, genome scan was conducted to map the ENU induced mutations. The mutation in one family exhibiting TGA was mapped to mouse chromosome 2, another family with phenotypes reminiscent of Holt-Oram syndrome was mapped to chromosome

4. A third family exhibiting phenotypes reminiscent of DiGeorge syndrome was found a mutation in semaphorin 6D (T7C). A fourth family exhibiting PTA with IAA had a semaphorin 3C (L605P) mutation. A fifth family with conotruncal malformation had a connexin43 (W45X) mutation. These studies show ENU mutagenesis together with ultrasound phenotyping provides an effective and high throughput method for recovering novel mutations causing congenital cardiovascular anomalies.

Association of Osmotic and Oxidative Stress. Z. Zhang[1], N. I. Dmitrieva[1], J. H. Park[2], R. L. Levine[3], and M. B. Burg[1]; [1] Laboratory of Kidney and Electrolyte Metabolism, [2] Laboratory of Biochemistry, [3] Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research.

Urea and NaCl are elevated in the renal inner medulla. We now find that high urea or NaCl increases reactive oxygen species in mouse renal inner medullary (mIMCD3) cells in culture. Previously, high NaCl, but not high urea, was found to cause DNA double strand breaks. We now tested whether high urea or NaCl causes oxidative damage to DNA or cellular proteins. We find that high urea increases mIMCD3 cell DNA single strand breaks and 8-oxoguanine lesions. High NaCl does not cause detectable 8-oxoguanine lesions. High urea or NaCl also greatly increases carbonylation of proteins in mIMCD3 cells. Carbonylation occurs within 5 minutes and with as little as 5 mM urea, a normal plasma level. It increases as urea is raised over the range in uremia. High raffinose increases ROS and carbonylation. High sorbitol and glycerol do not increase ROS or carbonylation. Carbonyl content is high in mouse renal inner medullas where interstitial NaCl and urea concentrations are normally high. There, numerous proteins are carbonylated and carbonylation occurs in both collecting ducts and thin limbs. Conclusions: 1) oxidative stress, associated with high urea, causes 8-oxoguanine DNA lesions in mIMCD3 cell DNA. 2) high urea or NaCl carbonylates proteins in mIMCD3 cells and in renal inner medullary cells in vivo. 3) In mIMCD3 cells a normal plasma concentration of urea causes carbonylation, and carbonylation increases over the uremic range of urea concentration, indicating that urea can contribute directly to the carbonylation found in uremia.

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